Histol Histopathol (2010) 25: 177-187
DOI: 10.14670/HH-25.177

http://www.hh.um.es

Histology and Histopathology
Cellular and Molecular Biology

Expression and distribution of the intermediate filament protein nestin and other stem cell related molecules in the human olfactory epithelium

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Summary. The olfactory epithelium (OE) is unique in regenerating throughout life and thus is an attractive target for examining neurogenesis. The nestin protein was shown to be expressed in the OE of rodents and is suggested to be essentially involved in the process of regeneration. Here we report the expression and distribution of nestin in the human OE at RNA and protein level. Moreover, we analysed the expression profiles in dependence on age and olfactory capacity. After sinus surgery, biopsies were taken from the olfactory epithelium of 16 patients aged 20-80 years with documented differences in their olfactory function. Our studies revealed that nestin is constantly detectable in the apical protuberances of sustentacular cells within the human OE of healthy adults. Its expression is not dependent on age, but rather appears to be related to the olfactory function, as a comparison with specimens obtained from patients suffering either from persistent anosmia or hyposmia suggests. Particularly, in the course of dystrophy, often accompanied with impaired olfaction, nestin expression was occasionally decreased. Contrarily, the expression of the p75-NGFR protein, a marker for human OE basal cells, was not altered, indicating that at least in the tested samples olfactory impairment is not connected with abnormalities at the basal cell level. These observations emphasize an essential role of nestin for the process of regeneration, and also highlight this factor as a candidate marker for sustentacular cells in the human olfactory epithelium.

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Key words: Human olfactory epithelium, Nestin, Sustentacular cells, Immunohistochemistry

Introduction

The olfactory epithelium (OE) exhibits a unique feature among mammalian neural systems, retaining the ability to regenerate throughout adult life. The procedure of neurogenesis has been extensively studied *in vivo* and *in vitro* in rodents, as well as in other species (Graziadei and Graziadei, 1979; Calof et al., 1998; Mackay-Sim and Chuahb, 2000; Schwob, 2002).

The olfactory epithelium is a pseudostratified structure consisting of three major cell types, the olfactory sensory neurons (OSNs), the non-neuronal sustentacular cells in the apical part of the epithelium, and the proliferating basal cells located adjacent to the basal membrane (Graziadei, 1971). Studies on rodent OE have shown that the latter undergo continuous mitotic activity and contribute to regeneration, achieved by differentiation into either neuronal or non-neuronal cells (Mackay-Sim and Chuahb, 2000; Jang et al., 2003; Carter et al., 2004; Chen et al., 2004). The perikarya of newly born receptor neurons then migrate towards the middle layer of the OE, profoundly changing their morphology within distinct differential stages (Morrison and Costanzo, 1992).

In order to understand the mechanisms underlying development and regeneration numerous studies have been performed, extensively characterizing the individual cell types within the rodent OE in terms of molecular and cellular composition. However, due to difficult access only a limited number of studies have so far investigated the histomorphology of the human OE

(Arnold et al., 2001; Bianco et al., 2004; Hahn et al., 2005). Several techniques of OE biopsy have been developed to obtain intact pieces of epithelia without permanent damage to the donor site (Lanza et al., 1994; Feron et al., 1998; Jafek et al., 2002; Lane et al., 2002). Prior studies have already noted some differences in immunoreactivity and structure between the rodent and the human OE (Hahn et al., 2005), indicating that neurogenesis might differ between both species. To better understand differences concerning processes, including birth and differentiation of neural cells in either species, the necessity for identifying cell type specific markers substantially increases.

In rodents, at least two populations of basal cells have been morphologically defined which reside in the olfactory progenitor layer: the globose basal cells (GBCs) and the horizontal basal cells (HBCs) (Andres, 1965; Graziadei and Graziadei, 1979). Both can be distinguished by specific markers, GBC-1 for GBCs (Goldstein and Schwob, 1996) and cytokeratin for HBCs (Calof and Chikaraishi, 1989; Yamagishi et al., 1989), although definite support for either of them as the ultimate olfactory stem cell is controversial. Contrary to rodents, in the human OE a morphological discrimination among basal cells is not possible (Hahn et al., 2005). However, the level distribution of p75-NGFR suggests that in humans just one common progenitor exists. Moreover, these cells are hardly distinguishable in shape from other cell types in the residual part of the epithelium.

For immature and mature OSNs molecular markers such as GAP43 (immature) and the olfactory marker protein (OMP) have been established (Margolis, 1982; Baker et al., 1989) that are useful for both species. In humans, maturating OSNs are dispersed throughout the middle part of the OE; in rodents, however, there is a columnar organization, with immature neurons more basal and mature OMP-positive neurons more apically oriented.

For sustentacular cells SUS-1 and SUS-4 have been described to be useful antibodies (Hempstead and Morgan, 1983, Goldstein and Schwob, 1996). Recently, nestin immunoreactivity has been detected in sustentacular cells in the OE of adult rats (Doyle et al., 2001). Nestin belongs to a highly diverse family of cytoskeletal factors and forms the VI class of intermediate filament proteins (Lendahl et al., 1990). For a long time the protein was assumed to exclusively mark neuronal progenitors of the central nervous system. Doyle and coworkers described its significant expression in sustentacular endfeet which are intimately associated with the basal lamina. Interestingly, nestin immunoreactivity was upregulated postbulbectomy and redistributed to the more apical part of sustentacular cells e.g. the cell bodies (Doyle et al., 2001). Thus, as a hypothesis, nestin is involved in cellular reorganization after damage and/or physiological replacement. This prompted us to elucidate the expression and distribution of nestin at the protein and mRNA level within the human OE. Thereby we intended to highlight similarities and differences compared to the rodent OE. Furthermore, we analyzed the expression pattern of nestin and some other markers depending on the age and the olfactory capacity of different donors. In addition to psychophysical examinations the application of immunohistochemistry-based investigations is increasingly discussed as a potential diagnostic tool for olfactory disorders, as well as their peripheric reasons. Impaired olfaction (e.g. anosmia, hyposmia) is a prevalent phenomenon (Landis and Hummel, 2006) often caused by sinonasal diseases (Damm et al., 2004). For the understanding and treatment of such diseases a profound knowledge of the basic characteristics of the human OE in terms of molecular and cellular composition is essential.

Materials and methods

The study was performed according to the Declaration of Helsinki on Biomedical Research Involving Human Subjects. It was approved by the Ethic Committee of the Ruhr University of Bochum (register number 2918). Patient data including all relevant information are listed in Table 1.

Biopsy of OE

OE biopsies were obtained with informed consent from subjects during sinus surgery or septoplasty. Subjects with major medical illnesses, including psychiatric illnesses and metabolic diseases (diabetes, renal insufficiency) were excluded. Patients with polyposis were also excluded. 16 subjects, aged 20-80

Table 1. Patient characteristics.

Patient-ID	Age	Gender	Diagnosis	Olfactory condition	Site of biopsy
1	80	M	Post mortem	unknown	Regio olfactoria
2	65	M	Post mortem	unknown	Regio olfactoria
3	35	M	SD	Hyposmia	NS, MT
4	50	M	SD	Normosmia	NS, MT
5	70	F	IA, SD	Anosmia	NS, MT
6	80	M	SD, CRS	Normosmia	NS, MT
7	80	M	CRS	Hyposmia	MT
8	21	M	CRS	Hyposmia	MT
9	20	F	SD	Normosmia	NS, MT
10	20	F	IA, SD	Anosmia	NS, MT
11	52	F	IA, SD	Anosmia	NS, MT
12	65	M	IA, CRS	Anosmia	NS, MT
13 *	-	M	CRS	Hyposmia	NS, MT
14 *	78	M	CRS	Hyposmia	NS, MT
15	48	M	CRS	Hyposmia	NS, MT
16	65	M	CRS	Hyposmia	NS, MT
17	41	M	SD	Anosmia	NS, MT
18	48	M	SD	Hyposmia	NS, MT

SD: Septal deviation, IA: idiopathic anosmia, CRS: chronic rhinosinusitis, NS: Nasal septum; MT: Middle turbinate; *: patients with Parkinson's disease.

years, participated in OE biopsy. For morphological and molecular biology purposes, up to four biopsies were taken from each patient including the area of the lateral superior wall of the nasal cavity, close to the radix of the medial turbinate and the opposite dorsal septum, as described before (Leopold et al., 2000). No biopsies were obtained from the nasal cleft immediately below the lamina cribrosa. From most subjects three samples were immediately fixed in 4% formalin (for immunohistochemistry), and one sample was immersed into RNAlater solution (QIAgen, Hilden, Germany) for mRNA analysis.

Psychophysical studies

All subjects were evaluated concerning their olfactory capacity by means of a standardized psychophysical test using "Sniffin' Sticks". Olfactory deficits were assessed by investigating the ability for odor identification, discrimination and detection-threshold sensitivity according to Hummel et al. (2007).

Preparation of post-mortem OE tissues

Post-mortem OE tissues were obtained from two subjects by the Department of Anatomy at the University of Aachen, Germany. The olfactory function of both subjects, aged 65 and 80 years, was unknown. At autopsy, the OE, bony septae, and contiguous cribriform plate were removed en bloc and fixed for 24 to 36 hours in 10% neutral buffered formalin. Subsequently, the samples were decalcified for three weeks in distilled water containing sodium ethylene-diamine-tetra-acetic acid, sodium hydroxide, and glycerol at pH 7.1–7.4. Tissue blocks were then cut into coronal blocks, dehydrated in graded ethanol solution and xylene, and embedded in paraffin (Trojanowski et al., 1991; Smutzer et al., 1998).

Antibodies

Immunolocalization studies were performed using monoclonal antibodies against the olfactory marker protein (OMP, Wako Chemicals, 1:5000), nestin (Chemicon, 1:50), beta-III-tubulin (Acris, 1:60), and p75-NGFR (Sigma, 1:500).

Immunohistochemistry

Probes were immediately fixed in 10% formalin and embedded in paraffin. Sections were cut at a thickness of 4 μm. Serial 4-μm sections were placed on slides, deparaffinized, rehydrated, and dried overnight at 37°C as previously described (Witt et al., 2009). Immunohistochemistry was performed using the peroxidase-antiperoxidase method in combination with 3-3'-diaminobenzidine (DAB). For standard DAB detection, endogenous peroxidase activity was quenched using 3% H_2O_2 diluted in distilled water. Subsequent to blocking in 1x Tris-buffered saline (TBS) containing 10% appropriate normal serum, sections were incubated overnight at room temperature with the primary antibody diluted in TBS containing 1% of normal serum. For secondary antibody reactions, the sections were washed in phosphate-buffered saline (PBS) and treated with either rabbit-anti-goat or goat anti-mouse biotinylated IgG antibodies (Dako, Copenhagen, Denmark, diluted 1:400 in PBS) for one hour at room temperature. Primary antibodies were then detected using a streptavidin-biotin-peroxidase kit (ZytoChem HRP Kit, Zytomed Systems GmbH, Germany) and visualized by DAB reaction. Performing sequential double immunostaining, binding of the first antibody was visualized by a brown reaction product generated using the DAB chromogen solution. To detect the second primary antibody, the AEC (3-amino 9-ethylcarbazole) chromogen (ZytoChem HRP Kit, Zytomed Systems GmbH, Germany) was used, yielding a red product. For better visualization of the nuclei some sections were counterstained with haematoxylin. The following controls were performed: (1) omission of the primary antibody in order to rule out nonspecific binding of the secondary antibody; and (2) in-parallel stainings of tissues (e.g., mouse and rat as well as normal human olfactory epithelium) previously reported to be immunopositive for the markers tested (Heilmann et al., 2000; Weiler and Benali, 2005). The summary of all immunohistochemical analyses is shown in Table 2. Staining intensities were rated according to the visual appearance in comparison to the slides and are indicated by "-" (no staining = 0), "+" (weak staining = 1), "++" (strong staining = 2), or "+++" (strongest staining = 3). Depending on the olfactory function two groups were built: group 1 consists of norm- and hyposmic patients and group 2 of anosmic patients with no olfactory function. The results were then compared using the chi-square (χ^2) test.

Table 2. Results of Immunohistochemistry.

Patient-ID	Olfactory condition	p75-NGFR	beta-III-T	Nestin	OMP
1	Unknown	++	+++	+++	+++
2	Unknown	++	++	+++	+++
3	Hyposmia	++	++	++	++
4	Normosmia	+++	++	+++	+
5	Anosmia	++	++	+	+
6	Normosmia	++	++	+	+++
7	Hyposmia	++	++	+	++
8	Hyposmia	++	++	++	+++
9	Normosmia	++	++	+++	+++
10	Anosmia	++	++	-	+
11	Anosmia	++	++	+	+
12	Anosmia	++	++	+	+
13	Hyposmia	++	++	++	++
14	Hyposmia	++	++	++	++
15	Hyposmia	++	++	+	++
16	Hyposmia	++	++	++	++
17	Anosmia	++	+	+	+
18	Hyposmia	++	++	+	++

RT-PCR analysis

For reverse transcription-PCR (RT-PCR), total RNA was isolated from olfactory mucosa (RNeasy Mini Kit, Qiagen, Hilden, Germany). The quality of the prepared RNA samples was assessed by A_{260}/A_{280} measurements and agarose gel electrophoresis. Equal amounts of RNA were reverse-transcribed using reverse transcriptase (Fermentas RevertAid cDNA synthesis kit; Fermentas GmbH, St.Leon-Roth, Germany) with oligo-p(dT)15 primers and random hexamer primers following the manufacturer's protocol in a final volume of 20 μl. The PCR was performed by using Taq-polymerase (Ampliqon, Copenhagen, Denmark) and gene-specific primers synthesized according to published cDNA sequences of the olfactory marker protein (OMP), nestin, p75-NGFR, and beta-III-tubulin. As an endogenous control we used GAPDH, a housekeeping gene that is highly expressed in human tissues.

Reactions were run on a PCR Cycler (Techne TC 512, Burlington, NJ, USA) under the following cycling conditions: 94°C for 2 min followed by 35 cycles of amplification. Each cycle included 30 s denaturation at 95°C, 30 s annealing at 62°C (except for b-III-tubulin: 58.5°C) and 30 s synthesis at 72°C. Amplified PCR-products were visualized after electrophoresis in 1.5% agarose gel containing ethidium bromide. The specificity of the PCR amplificates was confirmed by DNA sequencing.

Results

From a total of 42 biopsies taken from the presumptive olfactory area, 27 specimens (64%) contained olfactory epithelium. In all other biopsies regular or metaplastic respiratory epithelium was observed independent of the age and the olfactory condition. Immunohistochemical analysis was performed with OE biopsies of sixteen patients with ages ranging from 20-80 years. Specimens of a control group of healthy persons were compared with patients suffering either from persistent anosmia or hyposmia. One healthy subject was also subjected to RT-PCR analysis together with seven other patient samples to validate gene expression on mRNA level.

In the post-mortem nasal sample from patient 1, OE was mainly detectable in the region of the superior turbinate and in the insertion of the middle turbinate. The largest continuous area containing OMP-positive cells had an extension of about 1.5 mm. In the second post-mortem sample (patient 2), functional olfactory mucosa with OMP-positive cells was observed in a small area of the middle turbinate. All other regions were covered with regular or dysplastic respiratory epithelium. In all biopsies olfactory sensory neurons were visualized by antibody-staining of the olfactory marker protein. As shown in Figure 1, age had no obvious impact on the morphology of the epithelia or on the number of OMP-positive cells, as a comparison between the OEs derived from a 20, a 50 and an 80 year old subject revealed (Fig. 1a-c). In all cases sensory neurons were equally distributed among the middle part of the epithelium. Thus, densities of OMP-positive cells in normosmic patients are comparable between young and older subjects. The OSN area is apically restricted by a mono cell layer of sustentacular cells, exhibiting a typical shape, and by the basal cell layer adjacent to the lamina propria, both devoid of staining. The transition to the respiratory epithelium (RE), indicated by an arrow in Figure 1c, is characterized by an abrupt lack of OMP-positive cells.

Contrary to these observations, a comparison between OMP-staining of the epithelia taken from patients with olfactory impairment with that of a healthy

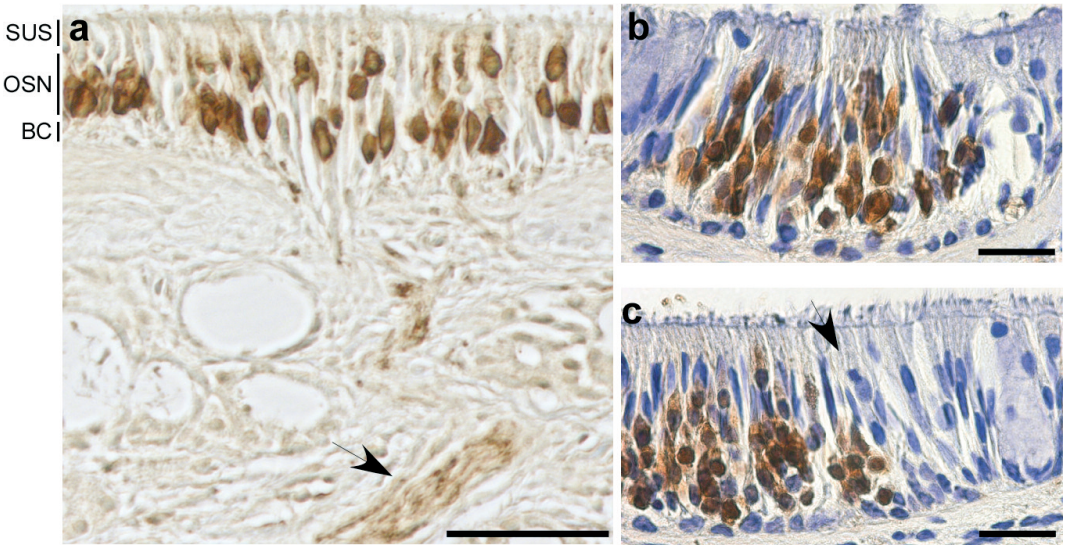

Fig. 1. Human olfactory epithelium (hOE) obtained from three patients with normosmia at the age from 20 (**a**, patient 9), 50 (**b**, patient 4) and 80 years (**c**, patient 6). Olfactory sensory neurons (OSNs) were stained with an OMP-antibody using the DAB procedure. In **(b)** and **(c)** cells were additionally counterstained by haematoxylin. The arrow in **(a)** indicates an olfactory nerve bundle. The arrow in **(c)** denotes the transition from olfactory to respiratory epithelium. SUS: sustentacular cells; OSN: olfactory sensory neurons; BC: basal cells. Scale bars: a, 50 μm; b, c, 20 μm.

person indicated that the amount of OSNs is dependent on the olfactory function (Fig. 2). While the epithelia of normosmic patients exhibited a high density of equally distributed OSNs (Fig. 2a), the number of OMP-positive cells was obviously decimated in that of anosmic persons (Fig. 2c).

For patients with designated hyposmia no strongly visible reduction in the number of OSNs could be observed (Fig. 2b). Application of the chi-square (χ^2) test revealed a significant reduction of OMP-positive cells in the OE of anosmics compared with hyp- and normosmics (Chi-square=13.2, df=2, p=0.001). For anosmics, these findings were partially accompanied by mild signs of dystrophia, which is expressed as a reduction in thickness of the epithelium, as well as by increasingly indistinct cell contours. However, most but not all OE biopsies from anosmic individuals strongly tended to a profound degeneration, as indicated by dysplastic morphology of the epithelium.

We next examined the presence and distribution of nestin in the human OE and its dependence on different olfactory functions. First, we concentrated on the expression profile of nestin in the OE of healthy subjects. The presence and localization of the protein was examined by immunostaining with a specific nestin antibody. Figure 3 shows the representative results of nestin staining in the olfactory epithelium of a normosmic subject. The protein was clearly detectable by persistent staining of the apical region of the OE where sustentacular cells are typically located. More precisely, counterstaining by haematoxylin illustrates that the microvillar fringe of the sustentacular cells is the location of major nestin expression (Fig 3a-c). In contrast, the bodies of the sustentacular cells showed no staining. Only poor signals were obtained in the basal cell layer where the proliferating neuronal progenitors reside. According to the observations described by Doyle and coworkers faint antibody reactions in this area might derive from staining of sustentacular endfeet (Doyle et al., 2001). The part of the OE where the OMP-

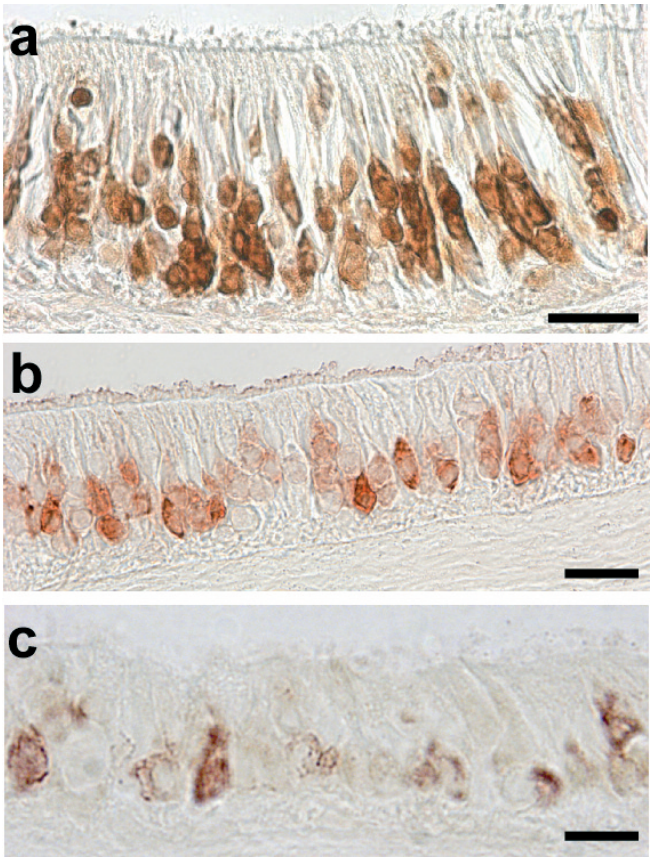

Fig. 2. OMP-staining of human OE biopsies derived from patients with different olfactory function. OSNs were stained with an anti-OMP antibody. The epithelium of a normsomic subject (**a**, patient 4) shows a high density of equally distributed OMP-positive OSNs. In comparison, in a hyposmic subject (**b**, patient 3) the number of OSNs is slightly reduced and the OE shows mild dystrophia. For an anosmic subject (**c**, patient 12) noticeable degeneration of the OE accompanied by a low density of OSNs was observed. Scale bars: 20 μm.

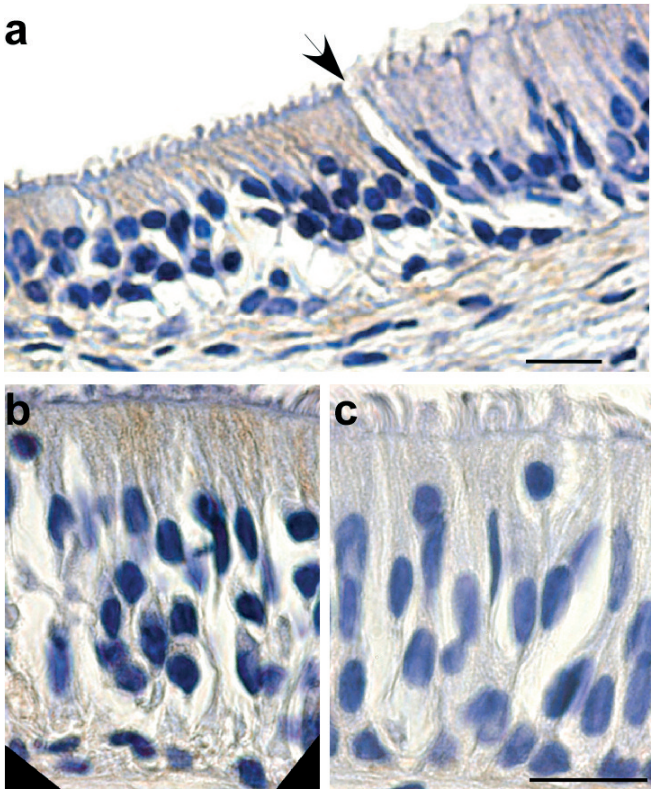

Fig. 3. Expression and distribution of the nestin protein in the human OE (patient 4). **a.** Nestin (DAB) is predominantly expressed in the apical processes of sustentacular cells of the OE, but absent in the respiratory epithelium (RE). The arrow indicates the transition between both epithelia. (Counterstaining with haematoxylin). **b-c.** Higher magnification of nestin-stained OE and RE (100x). Nestin expression appears in the apical part of the OE, while in the basal cell layer just weakly stained spots can be observed. Scale bars: 20 μm.

positive OSNs are located is exempt from nestin expression. Figure 4a shows the spatial separation from OSNs by parallel staining of nestin and OMP, in combination with a DAB and an AEC reaction, respectively. As similarly detectable for OMP-staining, the presence of nestin-positive cells abruptly interrupts at the transition to the respiratory epithelium, which is indicated by an arrow in the corresponding figure (Fig. 3a-c).

To further analyze the dependence of nestin expression on olfactory function, sections of the OE from normosmic, hyposmic and anosmic subjects were taken and compared concerning their staining pattern obtained by the antibody reaction (Fig. 4). Nestin immunoreactivity, which is clearly present in the sustentacular cell layer of normosmic patients, was slightly reduced in the OE of subjects suffering from hyposmia (cf Fig. 4a,b). A comparison of the expression patterns among eight hyposmic subjects revealed that

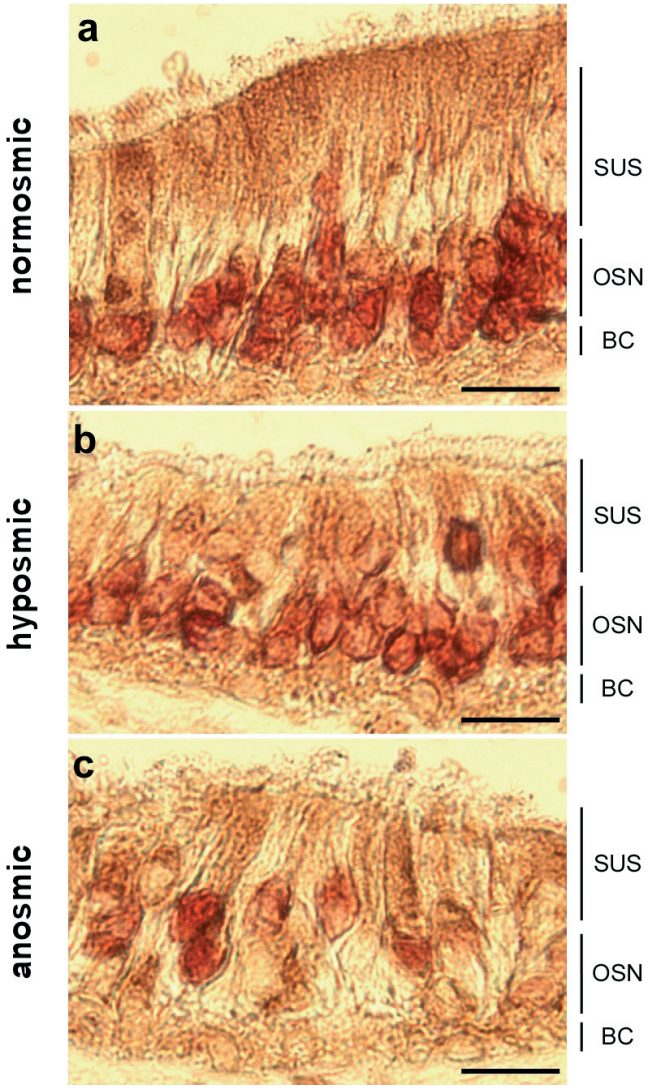

Fig. 4. Expression of nestin (DAB, brown) and OMP (AEC, red) protein in the OE of a normosmic (**a**, patient 9), hyposmic (**b**, patient 7) and an anosmic subject (**c**, patient 12). Nestin is clearly expressed in the sustentacular cells of the normosmic subjects and slightly reduced in the OE of hyposmic patients. In the case of anosmic patients protein expression varied between noticable reduction to complete lack of staining. SUS: sustentacular cells; OSN: olfactory sensory neurons; BC: basal cells. Scale bars: 20 μm.

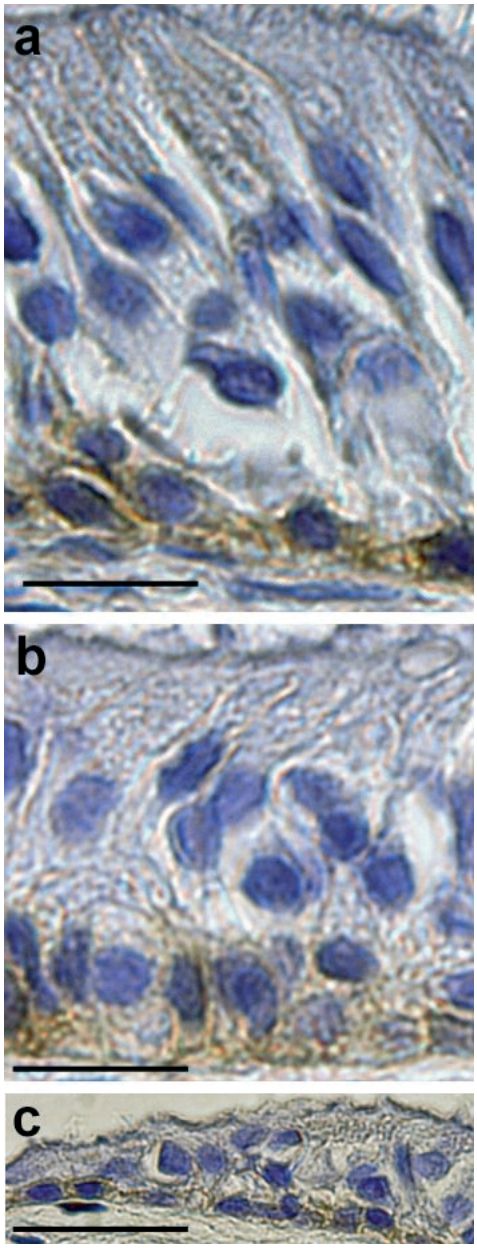

Fig. 5. Expression of p75-NGFR (DAB, brown) in the OE of normosmic (patient 4) **(a)**, hyposmic (patient 3) **(b)**, and anosmic (patient 11) **(c)** patients. In either case p75-NGFR-staining could be detected in the basal cells independent of the olfactory function. Counterstaining with haematoxylin. Scale bars: 20 μm.

staining intensities varied to some extent (cf Table 2), which also might be dependent on the prior location within the OE. In contrast, in the OE of anosmics, nestin expression was found to be noticeably reduced; at least in one specimen tested staining completely failed (Table 2, patient 10). However, due to subject dependent variations, differences between groups just missed the level of significance (Chi-square=5.67, df=2, p=0.059). Clearly visible contours of all cell types demonstrate that reduced staining was not due to a lack of the sustentacular cell layer which might have occurred at biopsy preparation. Due to strong interindividual differences concerning the morphology of the human OE, more patient samples will have to be analyzed to obtain definite evidence for the dependence of nestin expression on the olfactory function.

Degeneration of the OE, which often emerges as a reduction of thickness, and of OSN quantities might be a consequence of loss of basal cells or at least of impaired basal cell function. Therefore, we tried to identify differences at basal cell level within the OE of hyposmics and anosmics in comparison to that of normosmic subjects. For this reason we performed comparative immunostaining analysis on the epithelia of

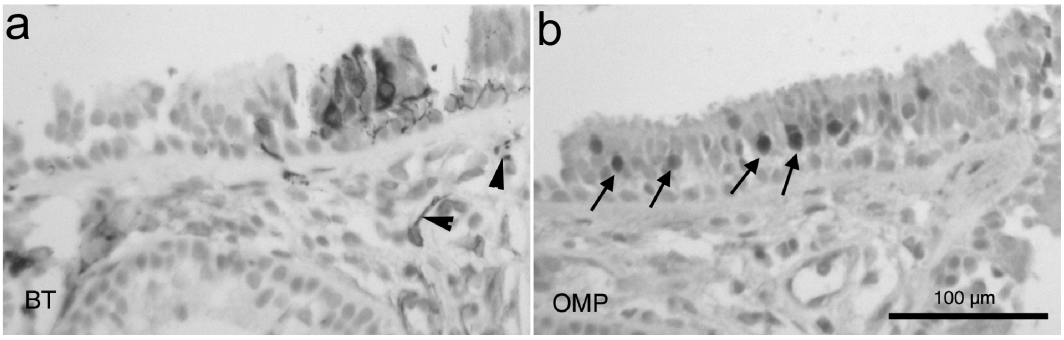

Fig. 6. Expression of beta-III-T in the OE of a hyposmic patient (Nr. 18): b-III-T antibody **(a)** labels not only mature OSN and its dendritic and axonal processes, but also non-mature OSN, as well as non-olfactory neurons, here probably trigeminal nerve fibers (arrowheads). **b.** lesioned olfactory epithelium with still intact OMP reactive cells (**b**, arrows). Scale bar: 100 μm.

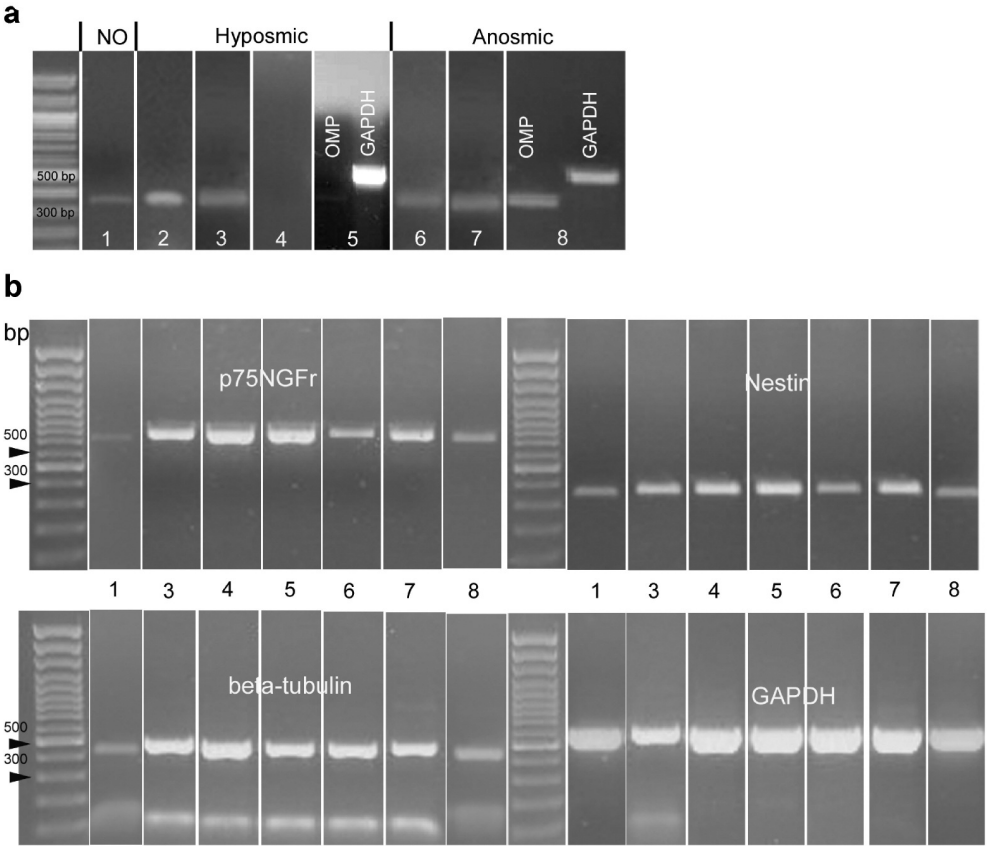

Fig. 7. a. RT-PCR amplification of OMP mRNA in normosmic (lane 1), hyposmic (lanes 2-5), and anosmic (lane 6-8) patients. Most of them showed a positive band for OMP gene products, except patient 4. Subject 5 shows only a very weak band (compared to overexpressed GAPDH signal). Even normosmic individuals presented relatively weak OMP bands. **b.** This survey illustrates the occurrence of mRNAs for several gene products associated with neurogenetic processes. mRNAs for p75-NGFR, nestin, and beta-III-tubulin are expressed in all subjects investigated.

Table 3. RT-PCR analysis of patients with different olfactory capacities.

Patient-ID	Olfactory condition	GAPDH	p75-NGFR	beta-III-T	nestin	OMP
5 *	Anosmia	+++	++	++	+	+
6 **	Normosmia	+++	+	+	+	+
13	Hyposmia	++	nd	nd	nd	+
14	Hyposmia	+++	+	++	+	+
15	Hyposmia	+++	++	++	+	-
16	Hyposmia	+++	++	++	+	+
17	Anosmia	+++	++	++	+	++
18	Hyposmia	+++	++	++	+	++

*: same patient as ID 5 in Table 2; **: same patient as ID 6 in Table 2

normosmics and of hyp- and anosmics with visible degeneration by using the p75-NGFR antibody. p75-NGFR was previously described as a marker of neuronal precursor cells in the human OE (Hahn et al., 2005). Interestingly, comparable intensities of p75-NGFR could be observed in all cases tested (Fig. 5). Thus, the basal cell layer seems not to be affected, arguing against a loss of basal cells as the reason for impaired olfaction in these patient cases. Concerning the expression of Beta-III-Tubulin (b-III-T) we could not see any difference between the groups. As shown in Figure 6a b-III-T expression was seen not only in mature OSN and its dendritic and axonal processes, but also in non-mature OSN, as well as in non-olfactory neurons. In parallel to the above described investigations, we performed RT-PCR analysis on patient samples, summarized in Table 3. Figure 7a shows the RT-PCR amplificates of OMP and the GAPDH housekeeping genes in total RNA extracted from the nasal mucosa of 8 subjects. We observed OMP bands of variable, although not quantified, intensities in all subjects, regardless of their olfactory capacity. Only one hyposmic individual did not show any amplificate for OMP-mRNA. Figure 7b compiles the results of gene products for the p75-NGF receptor (occurring in basal cells), beta-III-tubulin, and nestin. We observed all of these products in similar intensities.

Discussion

Impaired olfaction strongly influences quality of life; about 5% of the general population suffers from anosmia, which is characterized by complete loss of olfactory function (Hummel et al., 2007). Besides that, abnormalities like impaired olfaction, also described as e.g. partial anosmia or hyposmia, appear with high occurrence. Although the reasons for these diagnostic findings can be manifold and therefore often remain unknown, they mainly reveal a sinonasal nature (Hummel and Huttenbrink, 2005). To enhance our understanding of the particular molecular mechanisms of olfactory diseases it is essential to investigate the epithelial characteristics of certain patients with specific olfactory function.

Olfactory impairment at an older age is most likely due to a decreased secretion of the nasal mucus, and thus to a diminished mucociliary transport (Rawson, 2006). Studies that describe minimal age-related changes in the rodent OE at molecular, light microscopic and ultrastructural levels support the assumption that age-related loss in olfactory function is not an intrinsic feature of the aging process (Hinds and McNelly, 1981; Apfelbach and Weiler, 1991; Naguro and Iwashita, 1992). In contrast to that, for human beings increasing age significantly decreases the probability of obtaining olfactory tissue in biopsy material (Paik et al., 1992). At an age of about 60 years the OE becomes more frequently interspersed with patches of respiratory epithelium and is extensively displaced in elderly subjects. That is in line with our observations with detectable amounts of olfactory tissue in only 27 out of 42 samples. As regards this, differences between rodents and humans are probably based on the fact that these studies were performed with animals, which were not really old compared to the age humans reach.

Although we did no quantification, our results suggest that age has no influence on the number of OSNs, as a comparison of OMP-positive cells in the OE of young and elderly subjects revealed. Mature OMP-positive OSNs were similarly distributed among the major part of the OE. This is in contrast to findings from Paik et al. (1992), who reported decreased numbers of OSNs in elderly individuals. Contrary results might be explained by differences between autopsy material and surgical biopsies. On the other hand our data is in good agreement with a study of Feron et al. (1998), who did not observe age-related differences comparing various patient OEs.

Since techniques of OE biopsies were successfully established, several studies concentrated on the histological and histopathological examination of the human olfactory epithelium derived from subjects suffering from diseases associated with olfactory impairment, e.g. neurodegenerative diseases like schizophrenia or Parkinson's disease (Trojanowski et al., 1991; Crino et al., 1995; Arnold et al., 1998, 2001; Smutzer et al., 1998; Duda et al., 1999; Feron et al., 1999). More recent studies (Lee et al., 2000) analyzed the OE of anosmic patients with chronic sinusitis. In concert with their findings, we also observed a significant decrease of olfactory sensory neurons in the OE of all anosmic subjects tested. The identity of OSNs was proven by consistent detection of the olfactory marker protein by antibody staining. However, more quantitative assessment e.g. by RealTime-PCR was not performed as the aforementioned studies clearly demonstrated analogous findings. In morphological aspects, the orderly arrangement characteristic of the healthy OE was widely lost in most anosmics, demonstrating a degenerative appearance. In contrast, an obvious enhancement of OMP-positive cells in normosmics over hyposmics could not always be observed. To confirm these observations further analysis

of olfactory epithelia of additional hyposmic donors is indispensable. Kern (2000) showed signs of inflammation in some patients with chronic sinusitis. They mainly observed an infiltration of the OE with lymphozytes. In our patient biopsies we did not detect any inflammation of the olfactory mucosa as indicated by HE-staining of the basal lamina.

The major aim of our study was the analysis of the general expression pattern and the spatial distribution of the intermediate filament protein nestin within the human OE at protein and mRNA level. Nestin is known to be expressed by various cell types during development. However, its expression is usually transient and does not persist into adulthood, except for neural precursors of the central nervous system (Hockfield and McKay, 1985; Frederiksen et al., 1988; Lendahl et al., 1990; Fishell et al., 1993). Upon differentiation nestin is down-regulated and displaced by tissue-specific intermediate filament proteins (Lendahl et al., 1990; Sejersen and Lendahl, 1993). Thus, for a long time the protein was considered to be one of the best markers for neural progenitors in mammals (Lendahl et al., 1990; Chiasson et al., 1999; Rao, 1999). In 2001, Doyle and co-workers described the interesting finding that nestin is also expressed in the OE of adult rats. However, its expression generally was detected in the endfeet of sustentacular cells (SUS), which are located within the basal cell layer where neural precursors reside. More interestingly, after bulbectomy, when regenerative events probably get accelerated, nestin-positive staining significantly appeared in the apical part of the OE, where typically sustentacular cell bodies are located (Doyle et al., 2001). These findings strongly support an essential participation of nestin in regenerative processes of the rodent OE. The exact role of SUS cells has not been established in human OE so far. In rodents SUS cells have been thought to regulate the ionic composition of the mucus layer and to detoxify chemical olfactory stimuli (Getchell et al., 1984; Chen et al., 1992). In contrast to OSNs the population of SUS cells is not lost after unilateral olfactory bulbectomy. Exposing the OE to methyl bromide gas (MeBr) leads to an elimination of greater than 95% of SUS cells and OSNs, whereas a large portion of the basal cells is spared (Schwob et al., 1995; Schwob, 2002). Following this, a rapid regeneration of the SUS cells and OSNs is observed within weeks. It is presumed that SUS cells can be generated by globose basal cells of the rodent OE (Schwob, 2002).

For the human OE, RT-PCR analysis of olfactory mucosa already provided evidence for the expression of nestin at RNA level (Murrell et al., 2005). In concert with that, our immunohistochemical results clearly show that nestin is also expressed at protein level in the healthy human adult OE; nestin could be detected in all normosmic patients. However, the vast nestin-staining in the human OE is rather found in the typical microvillar protuberances of sustentacular cells than in the area of basal cells. At the basal lamina almost no staining was visible, most probably co-localizing with the endfeet of sustentacular cells. This hypothesis is based on findings from the aforementioned publication by Doyle and co-workers, where co-staining with the specific SUS-4 antibody uncovered these structures as the endfeet processes of sustentacular cells in the adult rat OE (Doyle et al., 2001).

Doyle and colleagues hypothesized that in rodents nestin might play a role in the migration of newborn olfactory neurons on the scaffolding of sustentacular cells, essentially taking part in the regeneration of the OE. Based on this assumption, we suggest that the human OE exhibits a generally more active state of regeneration than occurs in healthy rodents. Permanently generated OSN may then need guidance to reach their appropriate place in the epithelium, which could find support by a complex network of intermediate filament proteins such as nestin. This accompanies a similar hypothesis that was raised in the course of comparative expression analysis of p75-NGFR in the rodent and human OE (Hahn et al., 2005). Here, p75-NGFR revealed to be a suitable marker for proliferating basal cells in the human adult OE, which in turn was quite surprising due to the fact that in the rodent OE p75-NGFR is exclusively expressed during embryogenesis but not in adulthood (Vickland et al., 1991). Additionally, our observation of comparable p75-NGFR expression in the human adult OE of various aged donors is in good agreement with data provided by Hahn and colleagues (Hahn et al., 2005).

Contrary to the situation in rodents, nestin might be a suitable marker of sustentacular cells in the human OE, which is further supported by the lack of age-related differences in expression profiles in the OE of normosmic patients. In contrast, our results of comparative analysis of biopsy material taken from healthy persons with that derived from subjects with impaired olfaction (e.g. hyposmia, anosmia) suggest a dependence of the nestin expression on the olfactory function. Whereas for all hyposmics only weak reduction in nestin-expression was visible, antibody staining was more obviously reduced in the OE of the most anosmic donors; in one anosmic patient there was a complete loss of staining.

In some cases the OE of anosmic subjects showed strong degeneration, which was indicated by a dramatic decrease in thickness, accompanied by indistinct cell contours. It is unclear whether the detection of nestin was negatively affected here due to a lack of sustentacular cells. At least in the OE of some anosmics reduction of nestin staining cannot be attributed to cell degeneration as indicated by typical shapes of sustentacular cells. Unlike in rodents the human OE shows great intra- and interindividual varieties depending not only on the diagnosis but also on the site of biopsy. Therefore our results concerning the function-dependent nestin expression have to be interpreted critically. Taken together, the present study highlights new differences between the rodent and human olfactory

epithelium. Here we demonstrate permanent expression of the nestin protein in the apical part of sustentacular cells of the adult human olfactory epithelium, which is in contrast to rats. Moreover, the presented results provide a first indication for the dependency of the expression profiles of nestin mRNA and protein on the olfactory capacity.

Acknowledgements. We are greatful to Susane Kannabey and Kerstin Pehlke for excellent technical assistance and to Günter Gisselmann and Thomas Hummel for helpful discussion on the manuscript. This study was supported by the grant FoRUM (Nr. F596-07) from the Ruhr University Bochum.

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Accepted August 18, 2009