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Expression of *Hes6* and *NeuroD* in the Olfactory Epithelium, Vomeronasal Organ and Non-sensory Patches

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Abstract

Basic helix–loop–helix transcription factors *NeuroD* and *Hes6* promote neuronal differentiation. The expression of their genes in the olfactory epithelium (OE), vomeronasal organ (VNO) and the non-sensory patches of the posterior nasal cavity of mice was examined. As detected by *in situ* hybridization, *Hes6* was expressed in a basal progenitor layer of the embryonic OE. After birth, the expression of *Hes6* was detected in a cell layer above the basement membrane, globose basal cells (GBCs). Expression of *NeuroD* in the embryonic OE was in agreement with that previously described; and in the postnatal OE, it was detected in cells of GBC layer and cells upper to GBCs. In the VNO, *Hes6* was expressed throughout the sensory epithelium (S-VNO) at embryonic day 12, and later became restricted to a single layer of cells in the basal region of the S-VNO, where *Hes5*-expressing undifferentiated cells were present. *NeuroD* was expressed throughout the S-VNO during the embryonic stage. After birth, *Hes6* and *NeuroD* expressions were observed in the border between the S-VNO and non-sensory VNO. Immunohistochemistry using anti-*NeuroD* antibody revealed that *NeuroD*-positive cells were still present not only at the edges but also in the center of the S-VNO until P3. These findings suggest that *Hes6* and *NeuroD* are expressed in progenitors of chemoreceptor neurons and that the expression of *Hes6* precedes that of *NeuroD*. Moreover, in the regenerating VNO of bulbectomized mice, *NeuroD*-positive cells were observed both at the edges and in the center of the S-VNO, suggesting that neuronal turnover occurred in both regions. Moreover, in the dorsal fossa of the posterior nasal cavity, several non-sensory patches are formed between postnatal (P) days 10 and 21 because of programmed death of ORNs and GBCs. During embryonic stages, the expression of *Hes6* and *NeuroD* in the OE showed no regional differences. At P3–P7, expression of *NeuroD* and *Hes6* disappeared in the region corresponding to the presumptive non-sensory patches. The loss of these genes may stop the differentiation and may cause apoptosis of GBCs and ORNs.

Keywords: *Hes6*, *NeuroD*, olfactory epithelium, vomeronasal organ, *in situ* hybridization

Introduction

In the mammalian olfactory epithelium (OE), the olfactory receptor neurons (ORNs) continually die and are replaced by their progenitor cells. Basic helix–loop–helix (bHLH) transcription factors, i.e. *Mash1*, *Neurogenin1*, *NeuroD*, *Hes1*, *Hes5* and *Hes6*, are known to control the generation of progenitor cells and their differentiation of them to ORNs (Cau *et al.*, 1997, 2002; Guillemot, 1999; Bae *et al.*, 2000). *Mash1* is a determination gene for ORNs since *Mash1* null mutant mice fail to produce progenitor cells (Cau *et al.*, 1997). *Neurogenin1* is also determination gene, and is required for activation of genetic programs functioning downstream of *Mash1*. In *Neurogenin1* null mutant mice, progenitors of ORNs are generated but their differentiation is blocked (Cau *et al.*, 2002). *Hes1* and *Hes5* maintain progenitors in their undifferentiated form (Cau *et al.*, 2000). In contrast, *NeuroD* acts downstream of the above-mentioned genes and was demonstrated to have neuronal differentiation activity (Lee *et al.*, 1995). In the embryonic OE, as detected by *in situ* hybridization, *NeuroD* was expressed in the basal region (Cau *et al.,* 1997). Morphologically, the cell types of the embryonic OE are supporting cells, ORNs and basally located columnar-shaped cells (Cuschieri and Bannister, 1975). After birth, horizontal basal cells (HBCs) and globose basal cells (GBCs) are differentiated in the basal region. HBCs are keratin-positive cells and are in direct contact with the basement membrane. GBCs are devoid of keratin, lie above the HBCs and are generally accepted to be the progenitors of ORNs (Schwartz-Levey *et al.*, 1991; Suzuki and Takeda, 1991; Holbrook *et al.*, 1995; Huard *et al.*, 1998). In adult mice, *NeuroD* was detected by immunohistochemical means, in the layer superior to the GBCs, a postmitotic cell

layer (Nibu *et al.*, 1999). However, the same authors described that *NeuroD* expression, detected with the same antibody, was observed both in the basal and middle regions of the OE of mice during postnatal days (P) 1–28 (Nibu *et al.*, 2001). Therefore, it is not clear what cell type expresses *NeuroD* in postnatal and adult stages. Moreover, the recently cloned bHLH gene *Hes6* was shown to promote neuronal differentiation by inhibiting *Hes1* activity (Koyano-Nakagawa *et al.*, 2000). *Hes6* is known to be expressed in the embryonic OE (Bae *et al.*, 2000), but its detailed localization has not been investigated.

The chemoreceptor cells in the vomeronasal organ (VNO) also undergo continuous neurogenesis during development and after injury. The VNO consists of two epithelia: a thick sensory epithelium (S-VNO) that is located in the medial portion of the VNO and contains supporting cells and chemoreceptor cells at various stages of differentiation, and a thinner non-sensory epithelium (NS-VNO) that is located in its lateral portion. Unlike the OE, keratin-containing basal cells are not observed in the basal region of S-VNO (Witt *et al.*, 2002). The progenitor cells of chemoreceptor cells are localized at the boundary region between S-VNO and NS-VNO of adult mice (Barber and Raisman, 1978). Studies using the immunohistochemical detection of BrdU in rat and hamster VNOs have found progenitor cells not only in the margins, but also in the cells along the basement membrane, of the S-VNO (Ichikawa *et al.*, 1998; Weiler *et al.*, 1999; Martinez-Marcos *et al.*, 2000). Although *Mash1* expression in embryonic day 14.5 mice has been reported (Cohen *et al.*, 2000), little is yet known about the expression of other bHLH transcription factors in the VNO.

In the posterior nasal cavity of rodents, epithelial patches exclusively consisting of olfactory supporting cells and HBCs are found. Several such non-sensory patches were located among the OE as small patches, in the dorsal fossa of the first, second, third and fourth turbinates and corresponding septa (Suzuki *et al.*, 2000, 2001). The presence of Bowman's glands in the patches indicates the origin of the patches to be OE. In fact, in newborn mice, it was shown that normal OE occupied these regions and that the patches were generated by programmed cell death of ORNs and the progenitors, and by the subsequent disappearance of these cells during postnatal development (Suzuki *et al.*, 2000). Selective death during development has also been reported to occur in the VNO of mice: the NS-VNO contains neurons that disappear after birth for the formation of the respiratory epithelium (Tarozzo *et al.*, 1998; Cappello *et al.*, 1999). Therefore, it is not known whether the expression of bHLH transcription factors changes in the region where programmed cell death occurs. In the present study, we examined the expression of two differentiation factors, *NeuroD* and *Hes6*, in the OE, VNO and non-sensory patches of mice.

Materials and methods

Animals

Timed pregnant and adult ddY mice were obtained from Sankyo Laboratories. All animals were maintained in a heat- and humidity-controlled vivarium on food and water provided *ad libitum*.

Unilateral bulbectomy

Mice were anesthetized with Nembutal (Abbot Laboratories, North Chicago, IL), and unilateral bulbectomy was performed as described previously (Suzuki *et al.*, 1995). The bulbectomized mice were used at 12 days after surgery.

Tissue preparation

To obtain embryos, pregnant females were killed by cervical dislocation and their uteri with fetuses embryonic day (E) 10–18 carefully dissected out. Neonatal and adult mice were killed by an overdose of Nembutal given by intraperitoneal injection. The heads were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) overnight or with periodate–lysine–paraformaldehyde (PLP) for 4–6 h at 4°C. The specimen from adult mice were decalcified in 10% EDTA in Tris buffer (pH 7.6), and cryoprotected with 25% sucrose, embedded in OCT compound (TissueTek, Miles, Elkhart, IN), and frozen in a spray freezer (Oken, Japan). The tissues were sectioned coronally at a thickness of 8–10 μm. Sections were collected and placed on silane-coated slides.

RNA probes and *in situ* hybridization

cDNA fragments of *NeuroD*, *Hes6*, *Hes5* and *NCAM* were cloned by reverse transcriptase polymerase chain reaction (RT-PCR) using the total RNA extracted from the olfactory mucosa of adult mice and then used for the synthesis of cRNA probes. The sequences of the primers were 5′-ATGACCAAGGCGCGCCTAGA-3′ (55–74) and 5′-ACAGGACAGTCACTGTACGCAC-3′ (920–899; Genbank U28068) for *NeuroD*, 5′-ATGAGGTGCACACGTTCGTG-3′ (371–390) and 5′-GCGCAACTGTGTTACAAACG-3′ (1222–1203; Genbank AF260236) for *Hes6*, 5′-GGATGCTAATGAGGACGAGCG-3′ (63–83) and 5′-CAGCTTCATCTGCGTGTCGC-3′ (905–886; Genbank D32132) for *Hes5*, and 5′-CTACCCTCACCATCTACAACGC-3′ (376–397) and 5′-GACTGGGAGTCCTGGCCGAT-3′ (1354–1335; Genbank X15049) for *NCAM*.

The PCR was carried out for 35 cycles. Each resulting fragment was cloned into *Hin*dIII/*Eco*RI sites of pT7/T3 α18 (Ambion, TX) and sequenced. Digoxigenin (DIG)-UTP-labeled RNA probes were synthesized by use of an RNA transcription kit (Roche Diagnostics, Mannheim, Germany).

Sections were immersed in absolute ethanol for 5 min and in 0.2 N HCl for 20 min, and then washed twice in PBS for 5 min each time. Next, the sections were treated with

2 μg/ml of proteinase K (Takara, Kyoto) at 37°C for 15–20 min, washed in PBS, and refixed with 4% paraformaldehyde in 0.1 M phosphate buffer for 20 min. After having been washed twice in PBS, the sections were air-dried and hybridized. Hybridization was performed at 47°C for 16 h with a DIG-labeled RNA probe in a hybridization solution containing 50% formamide, 0.3 M NaCl, 0.02 M Tris–HCl (pH 8.0), 1 mM EDTA, 10% dextran sulfate, 1 × Denhardt's solution, 1 mg/ml yeast tRNA and 0.02% SDS. Hybridized sections were washed at 47°C in a solution containing 50% formamide and 2 × SSC for 1 h, and thereafter twice in 2 × SSC for 5 min each time. They were then treated with 20 μg/ml of RNase (Type II-A, Sigma Chemical Co., St Louis, MO) at 37°C for 30 min, and washed at 47°C in 50% formamide/2 × SSC followed by 50% formamide/1 × SSC for 1 h for each. After having been washed three times in PBS, the sections were incubated with 1% blocking reagent (Boeringer Mannheim GmbH, Mannheim, Germany) in maleic acid buffer (pH 7.5) for 1 h at room temperature. Subsequently, they were incubated overnight at 4°C with alkaline phosphatase-conjugated anti-DIG Fab fragments diluted 1:500 in PBS. After three washes in TBS, chromogenic reactions were carried out by using NBT/BCIP (Boeringer Mannheim).

Immunohistochemistry

A goat polyclonal antibody to *NeuroD* was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The sections were incubated with anti-neuroD antibody for 1 h at 37°C, and then stained by using a labeled streptoavidin–biotin (LSAB) kit (Dako, Kyoto). The immunoreactive product was colored by use of diaminobenzidine (DAB). Control reactions included: (i) PBS used instead of primary antibody, and (ii) primary antibody adsorbed with *NeuroD* peptide (Santa Cruz). The specificity of the antibody has been examined previously (Suzuki *et al.*, 2002).

Results

Olfactory epithelium

At E10, the olfactory placodes, which were oval-shaped epithelial patches, appeared in the anterolateral region of the head. *Hes6* was weakly expressed in the entire olfactory placodes and telencephalon (Figure 1A). At E12, the olfactory placode differentiated into the OE and the VNO. *Hes6* was expressed in the basal region of the OE and throughout the S-VNO, a thick sensory epithelium of the VNO (Figure 1B). Until birth, *Hes6* expression was observed in a single layer of cells just above the basement membrane (Figure 1C), termed basal progenitors. At E15, near the base of the septal wall, which corresponds to the presumptive septal organ of Masera, a group of *Hes6*-expressing cells was observed. The epithelium surrounding this patch was devoid of *Hes6* expression (Figure 1D). At the same time, nasal turbinates arose and developed as a series of elevated folds on the lateral wall. Strong expression of *Hes6* was observed in several layers of these developing turbinates (Figure 1E). After birth, *Hes6* was expressed in the cell layer above the basement membrane, GBCs. HBCs, which were directly against the basement membrane, were devoid of *Hes6* mRNA (Figure 1F). The signals of *Hes6* became weaker and were detected in scattered GBCs at P7. The expression pattern of *NeuroD* during embryonic stages was in agreement with that previously described (Cau *et al.*, 1997). After birth, it was expressed in GBCs and cells upper to GBCs (Figure 1G). The signals of *NeuroD* mRNA also became weak as the mice grew. Sense controls displayed no reactivity (Figure 1H).

Vomeronasal organ

At E12, the VNO appeared as a tubular structure. A thick epithelium of S-VNO and a thinner epithelium of NS-VNO could be distinguished. The expression of *Hes6* was detected throughout the S-VNO (Figure 1B). The expression of *NeuroD* was similar to that of *Hes6*. At E15, strong expression of *Hes6* was observed in the basal region of the S-VNO (Figure 2A). To examine whether cells in the basal region remain undifferentiated or enter a differentiation pathway, *Hes5* probe was used. *Hes5* is known to be a negative regulator to inhibit differentiation in the OE (Cau *et al.*, 2000). A few scattered cells were reactive with the *Hes5* probe (Figure 2B). *NeuroD* was expressed throughout the S-VNO (Figure 2C). In the NS-VNO, these genes were not expressed (Figure 2A–C). A marker of mature and immature ORN and VNO receptor cells, *NCAM*, was expressed in both S-and NS-VNOs. In the S-VNO, *NCAM* expression was detected throughout the epithelium except the basal layer. In the NS-VNO, a few *NCAM*-expressing cells were observed (Figure 2D). At P3, expression of *Hes6* was restricted to the border between the S- and NS-VNO (Figure 2E). At the same time, *NeuroD* was expressed also in this border region (Figure 2F).

Non-sensory patches

From E12 to P1, the expression of *NeuroD* and *Hes6* showed no regional differences. However, at P3, *NeuroD* expression disappeared from the dorsal fossa of the posterior nasal cavity (Figure 3A). At the same time, *Hes6* expression in that region was weak as compared with that in other regions (Figure 3B). The expression of *Hes6* disappeared from the dorsal fossa by P7. Immunohistochemical detection using anti-*NeuroD* antibody also failed to detect the immunoreactive cells in the dorsal fossa of P3 mice (Figure 3C). At that time, *NCAM* expression was present in both dorsal fossa and other regions (Figure 3D).

NeuroD expression after bulbectomy

Immunohistochemistry using anti-*NeuroD* antibody revealed that the expression pattern of *NeuroD* in the embryonic OE and VNO was similar to that of the *in situ* hybridization

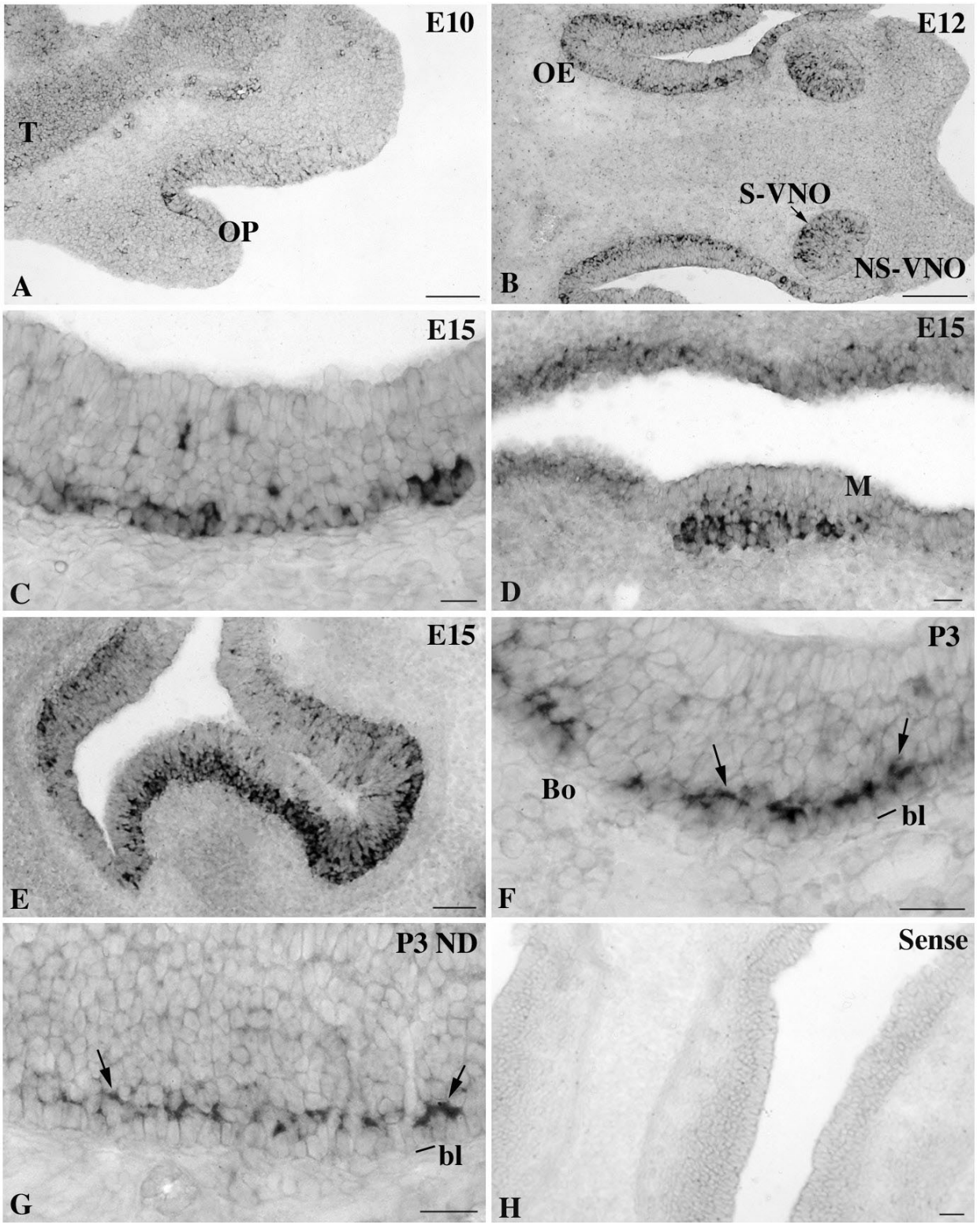

Figure 1 *In situ* hybridization with RNA probes for *Hes6* **(A–F)** and *NeuroD* **(G)** of coronal sections of mouse heads. (A) At E10, *Hes6* is expressed in the olfactory placode (OP). (B) At E12, *Hes6* is expressed in the olfactory epithelium (OE) and the vomeronasal organ (VNO). (C) At E15, *Hes6* expression in the OE is seen mainly in the basal region. (D) The patch consisting of *Hes6*-expressing cells is the Masera organ (M). E15. (E) Strong expression of *Hes6* is seen in the nasal turbinates. E15. (F) At P3, *Hes6* expression is seen in GBCs (arrows), which are located above the basal lamina (bl). Bo, Bowman's glands. (G) At P3, *NeuroD* expression is seen in GBCs and cells upper to GBCs (arrows). (H) Sense *Hes6* probe control. T, telencephalon. S-VNO, sensory VNO. NS-VNO, non-sensory VNO. bl, basal lamina. Bars 50 μm in A, B, E; 20 μm in C, D, F-H.

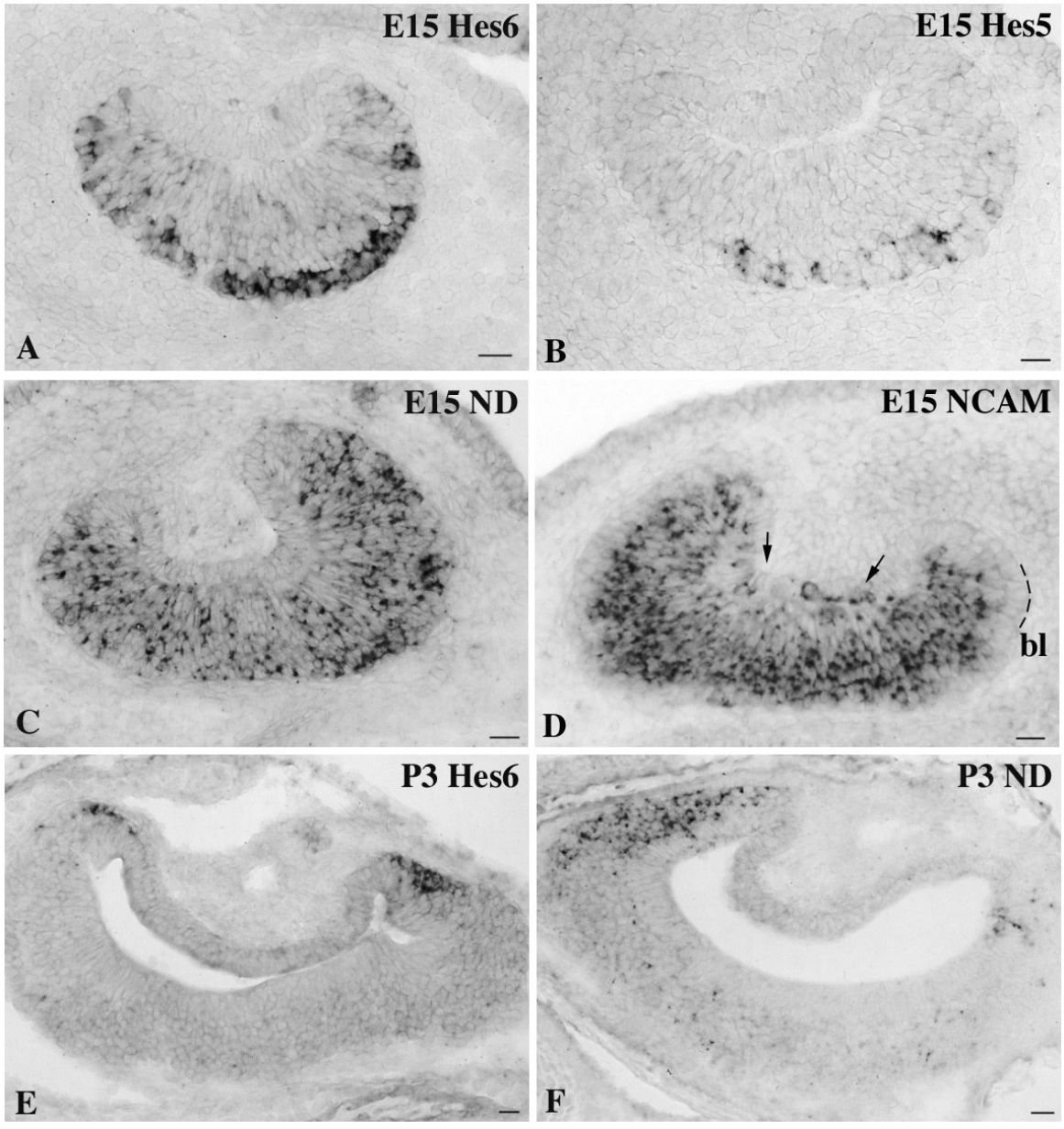

Figure 2 *In situ* hybridization with RNA probes for *Hes6* **(A, E)**, *Hes5* **(B)**, *NeuroD* **(C, F)** and *NCAM* **(D)** of coronal sections of the VNO. (A) At E15, *Hes6* expression is seen in the basal region. (B) At E15, *Hes5* expression is seen in scattered cells of the basal region. (C) At E15, *NeuroD* expression is seen throughout the S-VNO. (D) At E15, *NCAM* is expressed throughout the S-VNO except the basal region. *NCAM* expression is seen also in the NS-VNO (arrows). (E) At P3, *Hes6* expression is seen in the border between the S-VNO and NS-VNO. (F) At P3, *NeuroD* expression is seen in the border region. bl, basal lamina. Bars 20 μm.

data (not shown). In the stages of postnatal development and in the adult, when *in situ* signals were weak, immunoreactive cells could be detected. In the VNO, *NeuroD*-immunoreactive cells were observed both in the center and at the edges of the S-VNO at P3 (Figure 4A). During postnatal development, the immunoreactive cells gradually decreased in number and became restricted to the border between the S- and NS-VNO (Figure 4B). At 12 days after unilateral bulbectomy, *NeuroD*-immunoreactive cells in the margin of the S-VNO of the operated side (Figure 4C) were more abundant than those of the unoperated side (Figure 4B). Furthermore, *NeuroD*-immunoreactive cells appeared in the central region of the S-VNO (Figure 4C). In the OE, localization of *NeuroD*-immunoreactive cells was restricted to the basal region from P1 to adult, the number of immunoreactive cells decreased as development proceeds. A subset of *NeuroD*-immunoreactive cells was observed in the GBC region of adult mice; and after bulbectomy, *NeuroD*-immunoreacive cells were more abundant on the operated side than on the unoperated side (Figure 4D). In slides for

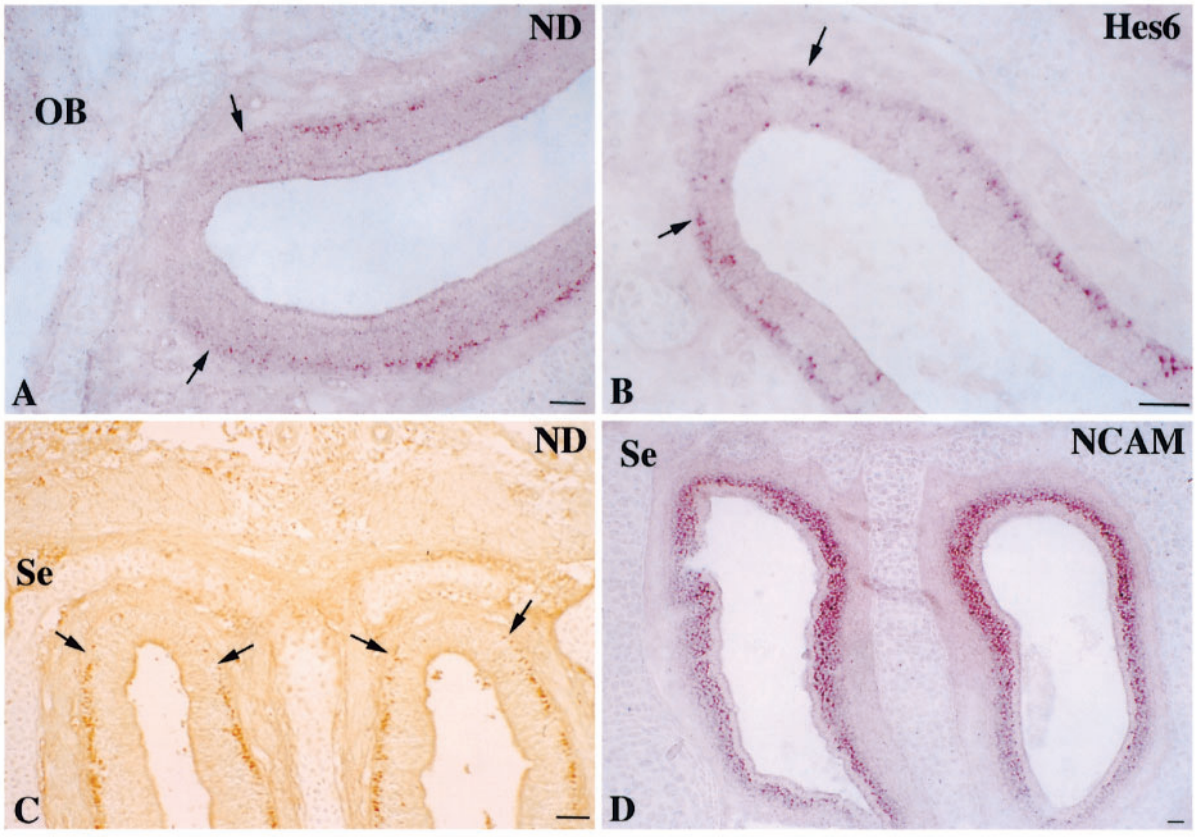

Figure 3 Formation of non-sensory patches in the posterior nasal cavity. P3 mouse. *In situ* hybridization with RNA probes for *NeuroD* **(A)**, *Hes6* **(B)** and *NCAM* **(D)**, and immunohistochemistry using anti-*NeuroD* antibody **(C)**. (A) Expression of *NeuroD* is absent in the dorsal fossa formed by the septum and the fourth nasal turbinate (between arrows). (B) Expression of *Hes6* is weak in the dorsal fossa formed by the fourth turbinate and lateral wall (between arrows). (C) *NeuroD*-immunoreactive cells are absent in the dorsal fossae of the 4th nasal turbinate (between arrows). (D) *NCAM* is expressed in the entire of olfactory epithelium including the dorsal fossa. OB, olfactory bulb. Se, septum. Bars 50 μm.

the two control reactions, i.e. PBS in place of primary antibody and preadsorbed primary antibody, the sections were completely unstained.

Discussion

In the embryonic OE, dividing progenitors are present in the apical and basal regions (Cuschieri and Bannister, 1975; Cau *et al.*, 2002). The present study clarified that *Hes6* was expressed in olfactory placodal cells and in basal progenitor cells of the embryonic OE and VNO. *Hes6* acts as a positive regulator of differentiation by inhibiting the action of bHLH-negative regulator, *Hes1* (Bae *et al.*, 2000). However, *Hes1* is expressed in apical side of the embryonic OE and is absent from the basal side. Another negative regulator, *Hes5*, in contrast, is expressed in the cells on the basal side of the OE (Cau *et al.*, 2000). It was confirmed also in the S-VNO of E15 that *Hes5* was expressed in scattered cells on the basal region. Therefore, the positive regulator *Hes6* and the negative regulator *Hes5* are co-expressed in the basal progenitor cells. In the embryonic OE, progenitors give rise to not only the ORNs, but also supporting cells, Bowman's glands and the cells outside the OE, i.e. LHRH neurons, ensheathing cells of the olfactory bulb and Schwann cells of olfactory axons (Farbman, 1992). In the postnatal OE, *Hes6* was expressed in cells of the GBC layer, suggesting its action is intrinsic to the ORN lineage. GBCs are identified as various cell-cycle marker-positive cells, [^{3}H]thymidine (Schwartz-Levey *et al.*, 1991), BrdU (Suzuki and Takeda, 1991), Ki67 and cyclin D1 (Ohta and Ichimura, 2001); and they differentiate into post-mitotic neurons. The present *in situ* hybridization and immunohistochemical study confirmed that *NeuroD* is expressed in GBCs, and post-mitotic neurons. In fact, Lee *et al.* showed that *NeuroD* was expressed not only in mitotic cells but also in post-mitotic cells of the developing central nervous system (Lee *et al.*, 2000). Moreover, Nibu *et al.* reported that *NeuroD*-immunoreactive cells were located in the layer superior to GBC and that after axotomy they were detected in higher layer in the regenerating OE than in the unlesioned side (Nibu *et al.*, 1999). However, in our data on regenerating OE, the number of *NeuroD*-immunoreactive cells increased, but their localization within the OE did not change.

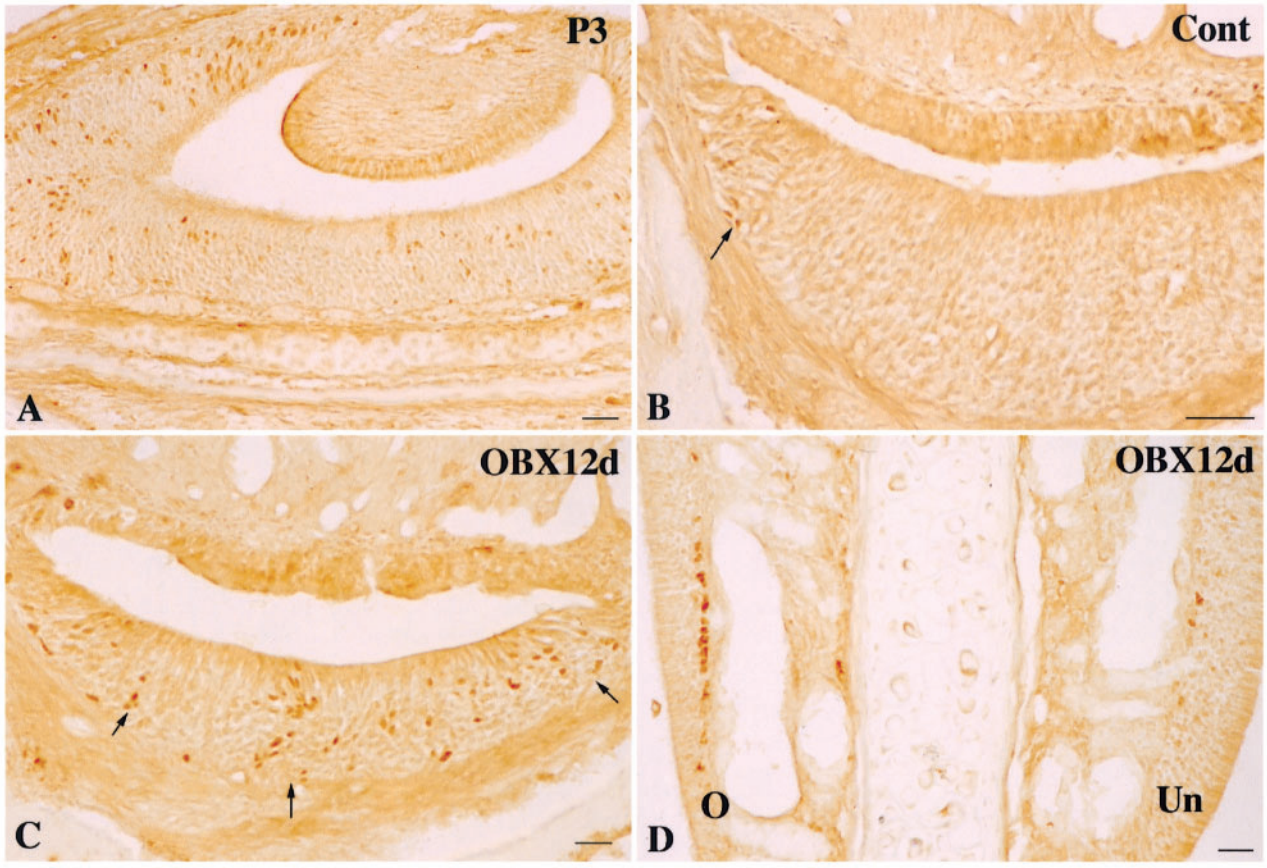

Figure 4 Immunohistochemistry using anti-*NeuroD* antibody in the VNO **(A–C)** and the OE **(D)**. (A) At P3, *NeuroD*-immunoreactive cells are present at the edges and in the central region of the S-VNO. (B) On the control side at day 12 after unilateral bulbectomy, a few *NeuroD*-immunoreactive cells (arrow) are seen at the edges of the S-VNO. (C) On the ipsilateral side at 12 days after unilateral bulbectomy, clusters of *NeuroD*-immunoreactive cells (arrows) are present at the edges and in the central region of the S-VNO. (D) At 12 day after unilateral bulbectomy. On the control side (Un), a subset of GBCs is immunoreactive for *NeuroD*. Many *NeuroD*-immunoreactive cells are seen in GBC layer on the ipsilateral side (O). Bars 50 μm.

In the VNO, the expression of *Hes6* and *NeuroD* in postnatal periods was similar to that of progenitors of chemoreceptor cells, i.e. the pool of dividing cells observed at the boundary between NS- and S-VNO. Also, *NeuroD*-immunoreactive cells appeared in the central region of regenerating S-VNO, where progenitor cells are present (Barber and Raisman, 1978; Ichikawa *et al.*, 1998; Weiler *et al.*, 1999; Martinez-Marcos *et al.*, 2000). It is believed that dividing cells at the boundary do not migrate to the central region of the S-VNO, and represent a pool for growth, whereas cells in the central region would participate in cell turnover (Jia and Halpern, 1998; Weiler *et al.*, 1999). However, abundant *NeuroD*-immunoreactive cells at the edges of the regenerating VNO revealed that cell turnover occurred in that region. Moreover, in the regenerating and embryonic S-VNO, *NeuroD*-expressing cells showed a vertically diffused pattern of localization. This may reflect the arrangement of chemoreceptor cells, for the mature chemoreceptor cells were diffusely distributed within the thick layer of chemoreceptor neurons, in contrast to the OE, where ORNs appeared more apically (Witt *et al.*, 2002).

During neurogenesis, bHLH genes are sequentially expressed as a result of activation cascade in which the early genes activate the expression of the late genes. The spatial pattern of expression of *Hes6* and *NeuroD* in the VNO and the OE suggests that expression of *Hes6* precedes that of *NeuroD*. *NeuroD* may activate downstream of *Hes6* in ORN and VNO receptor cell lineage. This expression pattern is also true in the developing retina: *Hes6* is expressed in both undifferentiated and differentiated cells (Bae *et al.*, 2000), whereas *NeuroD* is expressed in the differentiated population of retinal cells (Ahmad *et al.*,1998).

Our previous study showed that non-sensory patches were generated by programmed cell death of ORNs and their progenitor GBCs, and by the subsequent disappearance of these cells from P10 to P21 (Suzuki *et al.*, 2000). The present study clarified that expression of *Hes6* and *NeuroD* disappeared from the OE before apoptosis occurred. Moreover, the embryonic NS-VNO contains receptor cells (Tarozzo *et al.*, 1998) (see also this study), which disappear by apoptosis after birth to produce the respiratory epithelium (Cappello *et al.*, 1999). Neither *Hes6* nor *NeuroD* was expressed in the

embryonic NS-VNO. These genes might have been expressed there in earlier stages for a short time and then disappeared. Studies using bHLH gene-null mutant mice have shown that these genes regulate not only differentiation but also apoptosis. In *NeuroD*2-null mice, brain areas that would normally express *NeuroD*2 showed apoptosis (Olsen *et al.*, 2001). The endocrine pancreas of *BETA2*/*NeuroD*-deficient mice undergoes massive apoptosis and, consequently, animals die of diabetes shortly after birth (Naya *et al.*, 1997). In the present study, upstream genes, such as *Mash1* and *neurogenin1*, may also disappear from the presumptive non-sensory patches or NS-VNO before overt apoptosis. The loss of expression of these genes may stop the differentiation into ORNs or chemoreceptor cells and lead to apoptosis of these cells and their progenitors.

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