NOTE *Anatomy*

Transient appearance of the epithelial invagination in the olfactory pit of chick embryos

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ABSTRACT. In this study, immunohistochemical analysis has been performed using neuronal markers (GAP43, NCAM and PGP 9.5) to characterize the epithelial invagination in the medial wall of the olfactory pit in the chick embryos. At stages 26–27, the epithelial invagination was primarily composed of characteristic round-shaped cells, which were negative for neuronal markers. These cells were also found in the medial wall of the olfactory pit at stage 24, whereas the epithelial invagination was not observed at any stages other than stages 26–27. The possible relationship between the round-shaped cells and the migratory cells is discussed.
KEY WORDS: aves, development, immnohistochemistry, olfactory organs

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The presence of the anlage of the vomeronasal organ (VNO) in avian embryos is controversial. Some researchers claim that the anlage of the VNO is not present in birds. By other researchers, however, slight epithelial invagination in the medial wall of the olfactory pit has been considered to be the anlage of the VNO in the chick embryos [6]. Indeed, this epithelial invagination shows similar localization and similar shape to the anlage of the VNO of other animals like mammals. Furthermore, it exists only during the limited time of ontogeny and disappears as the development proceeds [6]. To date, development of the olfactory organ in the chick embryo has been investigated by many researchers [1, 3, 7, 11, 17]. However, little attention has been paid to the anlage of avian VNO in these literatures.

In rodents, a group of cells have been shown to migrate from the olfactory placode to the telencephalon during the early period of development. They include the terminal nerve (TN) cells, the gonadotropin releasing hormone (GnRH) neurons, the olfactory ensheathing cells (OEC) and the olfactory marker protein (OMP)-expressing cells [2, 4, 10, 19, 25, 27, 30–32]. Also, in the chick embryos, the development and distribution of the migratory cells have been intensively examined [12, 13, 16, 18, 20–23, 33]. However, to the best of our knowledge, no reports have mentioned the relationship between the migratory cells and the VNO anlage in the chick embryos.

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In this study, we used three neuronal markers: growth associated protein 43 (GAP43), neural cell adhesion molecules (NCAM) and protein gene product 9.5 (PGP 9.5), to investigate the epithelial invagination in the medial wall of the olfactory pit, the so-called VNO anlage of the chick embryo, in order to reveal its immunohistochemical properties and the possible relationship to the migratory cells.

Fertilized chicken eggs (*Gallus gallus domesticus*) were purchased from a local farm. The eggs were incubated at 38°C until appropriate developmental stages [15]. A total of 22 embryos from stage 22 to stage 29 were used in experiments. The heads were dissected from the embryos, fixed in Bouin's solution without acetic acid at 4°C overnight, routinely embedded in paraffin and cut coronally at 7 μm in thickness.

Immunohistochemistry was carried out using avidin-biotin peroxidase complex (ABC) method in the chick embryos at stages 26–27. Three primary antibodies were used as neuronal markers: rabbit anti-PGP 9.5 (1:1,000 dilution, UltraClone, RA95101, Wellow, U.K.), rabbit anti-NCAM (1:1,000 dilution, Millipore, AB5032, Billerica, MA, U.S.A.) and rabbit anti-GAP43 (1:3,000 dilution, Novus Biologicals, NB300-143, Littleton, CO, U.S.A.). The anti-PGP 9.5 antibody has been raised against PGP 9.5 protein purified from human brain and used previously in chicken tissues [5]. The anti-NCAM antibody has been raised against purified chicken NCAM, and its specificity has been confirmed by the manufacturer. The anti-GAP43 antibody has been raised against a synthetic peptide corresponding to a C terminal region of rat/mouse GAP43 and was used previously in chicken tissues [24, 26]. After deparaffinization, the sections were incubated in 0.3% H_2O_2 in methanol for 30 min at room temperature (RT) to inactivate endogenous peroxidase. The sections were incubated with 2% normal

donkey serum in phosphate-buffered saline (PBS, pH 7.4) for 30 min at RT to block non-specific binding. Then, the sections were incubated with one of the primary antibodies at 4°C overnight. Subsequently, the sections were incubated with biotinylated-donkey anti-rabbit IgG (1:1,000 dilution) for 1 hr at RT. Then, the sections were incubated with Vectastain ABC reagent (Vector Laboratories, Burlingame, CA, U.S.A.) for 45 min at RT. The sections were colorized with 0.05 M Tris-HCl buffer (pH 7.6) containing 0.01% 3-3′ diaminobenzidine tetrahydrochloride and 0.003% H_2O_2 at RT for 10–15 min. Finally, the sections were counterstained with hematoxylin. Between each step, sections were washed twice with 0.1% Triton-X 100 in PBS and once with PBS. All antibodies were diluted in 1% bovine serum albumin in PBS. Some of the sections (stages 22, 24, 26–27 and 29) were stained with hematoxylin-eosin (HE) for general histological examination.

At stages 26–27 (about 5 days of incubation), a pair of olfactory pit was situated in the rostroventral aspect of the head of chick embryos. At this time of development, it was not possible to distinguish the olfactory epithelium (OE) and the respiratory epithelium (RE) in the nasal pit. Slight invagination was seen in the medial wall of the olfactory pit (Fig. 1B). The invaginated area contained at least 2 types of cells: one was round-shaped cells which had a round nucleus with prominent nucleoli and relatively small, pale-staining cytoplasm, and the other was spindle-shaped cells which had an elongated nucleus (Fig. 1B'). In addition, a group of migratory cells were distributed along the olfactory nerve (ON) extending from the rostral part of the olfactory pit to the telencephalon (Fig. 1C and 1C').

Cells constituting the epithelium showed marked differences in immunohistochemical properties between the invaginated region and the remaining region of the nasal pit. The invaginated region was mainly composed of the cells negative for neuronal markers (NCAM, PGP 9.5 and GAP43), while most of the cells in the remaining region were positive for these markers (Fig. 2). Among the 2 types of cells distinguished in the invaginated region, the round-shaped cells were negative for all three neuronal markers (black arrowheads in Fig. 2A–2C), and the spindle-shaped cells were positive for the neuronal markers (black arrows in Fig. 2A–2C). Other than the invaginated region, spindle-shaped cells positive for neuronal markers were distributed in the epithelium (white arrows in Fig. 2D–2F). In addition, mitotic figures were observed in the superficial layer of the entire epithelium of the olfactory pit (open arrowheads in Fig. 2). These cells were negative for the neuronal markers.

At stage 24 (about 4 days of incubation), a few round-shaped cells similar to those described above were detected, although the invaginations were not clearly observed in the medial wall of the olfactory pit (Fig. 3A). Such cells were not found at stages 22 and 29 (about 3.5 days or 6 days of incubation, respectively) (Fig. 3B and 3C).

To date, development of the olfactory organ in mammals and birds has been extensively investigated morphologically and histologically [1, 3, 7–9, 12, 13, 17, 18, 20, 29]. However, no literatures have mentioned the accumulation

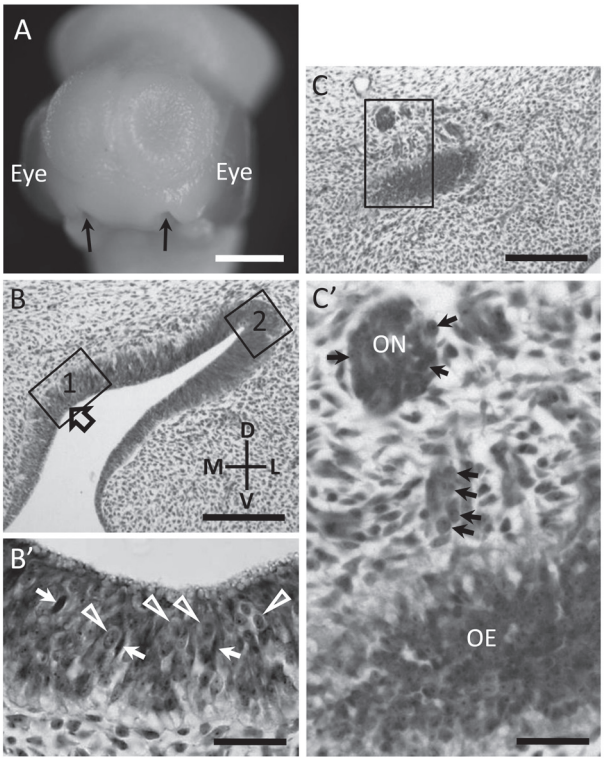

Fig. 1. The olfactory pit of the chick embryos at stage 27. (A) Frontal view of the head. Arrows indicate the olfactory pits. (B) HE-stained cross section through the olfactory pit. Dorsal is top, and medial is left. Open arrow in B indicates the slight invagination in the medial wall of the olfactory pit. (B') Higher magnification view of the invaginated area, showing round-shaped cells (open arrowheads) and spindle-shaped cells (white arrows). (C) A cross section at more rostral plane than (B). (C') Higher magnification view of the boxed area in C. The olfactory nerve (ON) extending from the olfactory epithelium (OE) toward the telencephalon. Note group of migrating cells (arrows) in close association with the ON. Boxed areas 1 and 2 in B indicate the regions shown in Fig. 2A–2C and Fig. 2D–2F, respectively. Scale bars: 1 mm in A, 100 μm in B and C and 20 μm in B' and C'.

of characteristic round-shaped cells in the medial wall of the olfactory pit in the chick embryos at stages 24–27 or in the embryos of other animals at their corresponding period. Thus, this is the first report of the observation of these round-shaped cells.

A group of cells migrating from the epithelium to the telencephalon along the olfactory nerves were mainly observed in the medial wall of the olfactory pit. Since the round-shaped cells in the medial wall of the olfactory pit were found only in the restricted region and restricted period of development and disappeared thereafter, it reminds us of the developmental process of the migratory cells derived from the olfactory placode. Multiple types of cells have been shown to migrate from the olfactory placode. One of them, the GnRH neurons, migrates from the medial wall of the olfactory pit to the telencephalon along the olfactory nerves in the chick embryos [21, 22]. The GnRH neurons distrib-

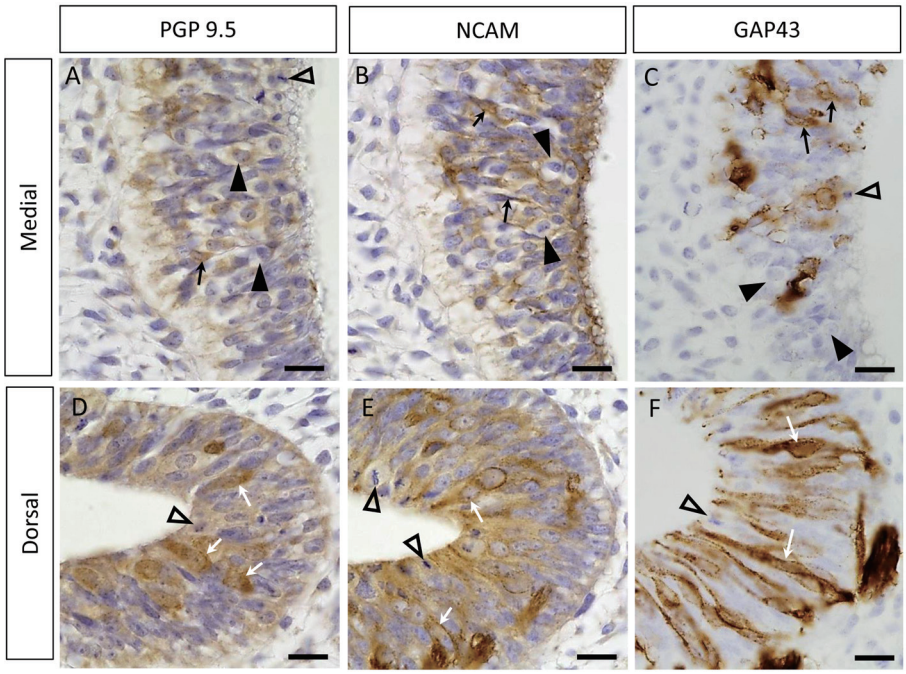

Fig. 2. Immunohistochemistry for neuronal markers: PGP 9.5, NCAM and GAP43. (A–C) Medial wall of the olfactory pit. Round-shaped cells with round nucleus surrounded by a relatively small cytoplasm (filled arrowheads) are negative for neuronal markers. Spindle-shaped cells (black arrows) are positive for neuronal markers. (D–F) Dorsal wall of the olfactory pit. Spindle-shaped cells (white arrows) are positive for neuronal markers. Open arrowheads indicate mitotic figures in the superficial layer of the epithelium. Scale bars: 10 μm.

uted in the olfactory pit, telencephalon and the mesenchyme between them are cells of spindle-shape and extend the cytoplasmic processes [20–22, 28]. According to Mulrenin *et al.*, the GnRH neurons in the chick embryos are detected for the first time in the olfactory placode at stage 19, then migrate into the mesenchyme and are no longer detectable in the epithelial lining of the olfactory pit by stage 35. Furthermore, in contrast to the number of GnRH neurons in the epithelium, which is relatively constant throughout development, those in the mesenchyme show a 10-fold increase from stage 25 to stage 26, suggesting a rapid transition from the epithelium to the mesenchyme immediately after the acquisition of GnRH peptide [20]. We speculate that a part of the round-shaped cells observed in the epithelial invagination are the precursors of the GnRH neurons before its onset of GnRH expression, and soon after they acquire the immunoreactivity for GnRH, they become spindle-shaped cells and migrate from the epithelium to the mesenchyme. The results reported by Mulrenin *et al.* support our hypothesis.

Of course, it is also possible that these round-shaped cells are related to the migratory cells other than the GnRH neurons. The OECs are another example of migratory cells derived from the olfactory placode [34]. In the chick embryos earlier than stage 20 (about 3 days of incubation), the olfactory nerves are negative for glial cell markers including microtubule associated protein (MAP4), but they become positive for the glial cell markers by stage 21 (about 3.5 days of incubation) [11]. From stages 23–24 (about 4 days of incubation), the OECs situated along the olfactory nerve increase their number as the development proceeds. By stage 34 (about 8 days of incubation), they enter the presumptive olfactory bulb along with the olfactory nerves [23]. In the mouse embryos later than E10.5 (correspond to stage 18 in the chick embryos), spindle-shaped OECs surround the migratory cells and olfactory nerves within the migratory cell populations emerged from the olfactory placode [2, 14, 19]. We speculate that a part of the round-shaped cells observed in the medial wall of the olfactory pit in this study might be the precursors of the OECs, and they subsequently change the cell shape and migrate from the epithelium to the mesenchyme.

The TN cells are also another migratory cell derived from the olfactory placode. In the rodent embryos, the TN cells emerge from the medial wall of the olfactory pit and provide the GnRH neurons the migratory route to the telencephalon [27]. However, it is not possible to discuss about the relationship between the TN cells and the epithelial invagination, as well as with the round-shaped cells included there, since markers to identify the TN cells in the chick embryos at stages 26–27 are not known to date.

We do not exclude a possibility that the round-shaped cells, as well as the spindle-shaped cells, in the medial wall of the olfactory pit differentiate into the cells constituting the OE or the RE. The OE and the RE were not distinguishable

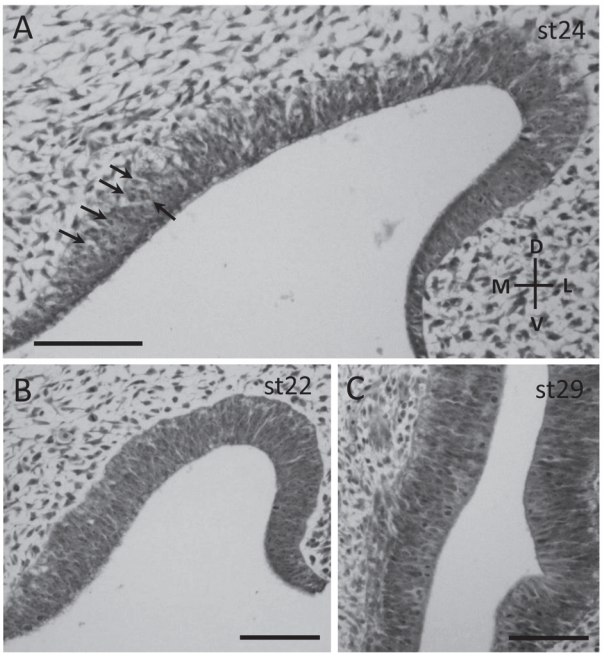

Fig. 3. HE- stained olfactory pit of the chick embryos at stages 24 (A), 22 (B) and 29 (C). Dorsal is top, and medial is left. Arrows indicate the round-shaped cells with small cytoplasm in the medial wall of the olfactory pit at stage 24. These cells are not observed at stage 22 and stage 29 (B, C). Scale bars: 50 μm.

in the olfactory pit at stages 26–27. They become distinguishable from one another by their morphological difference only after stage 29 in the chick embryo [17]. Therefore, it is not known whether the ventral region of the medial wall of the olfactory pit, where the round-shaped cells were situated at stages 24–27, differentiates into the OE or the RE at later stages.

Taken together, the present data demonstrate that the epithelial invagination observed in the medial wall of the olfactory pit in the chick embryo mainly consists of the round-shaped cells and a few spindle-shaped cells. Whether these cells differentiate into the migratory cells or the cells constituting the OE and/or the RE as the development proceeds should be determined in the future study. In the rodent embryos, epithelial invagination in the medial wall of the olfactory pit increases its depth to make the VNO separated from the nasal cavity. Appearance of the invagination in the olfactory pit of the chick embryos, in which the VNO will not develop, is intriguing, because it suggests multiple roles played by the VNO anlage.

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