Embryonic Pax7-Expressing Progenitors Contribute Multiple Cell Types to the Postnatal Olfactory Epithelium

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Prolonged neurogenesis driven by stem/progenitor cells is a hallmark of the olfactory epithelium (OE), beginning at the placodal stages in the embryo and continuing throughout adult life. Despite the progress made to identify and study the regulation of adult OE progenitors, our knowledge of embryonic OE precursors and their cellular contributions to the adult OE has been stalled by the lack of markers able to distinguish individual candidate progenitors. Here we identify embryonic OE Pax7⁺ progenitors, detected at embryonic day 10.5 (E10.5) in the olfactory pit with an antigen profile and location previously assigned to presumptive OE stem cells. Using Cre-loxP technology (Pax7-cre/ROSA YFP mice), we expose a wide range of derivatives, including CNS and olfactory neurons, non-neuronal cells, and olfactory ensheathing glia, all made from embryonic Pax7⁺ cells. Importantly, the expression of Pax7 in the embryonic OE is downregulated from E15.5, such that after birth, no Pax7⁺ cells are found in the OE, and thus the progenitor population here identified is restricted to embryonic stages. Our results provide the first evidence for a population of Pax7-expressing embryonic progenitors that contribute to multiple OE lineages and demonstrate novel insights into the unique spatiotemporal patterning of the postnatal OE.

Introduction

The olfactory epithelium (OE) is one of a few exclusive regions in the adult nervous system where lifelong neurogenesis occurs (Graziadei and Graziadei, 1979; Schwob, 2002). Neurogenesis and maintenance of the sense of smell is dependent on the biological responses of stem and/or progenitor cells. A common problem in stem cell biology however, is the limited availability of markers capable of identifying precursor cells whose ontogeny, *in vivo* niche, and lineage potential can be examined (Weissman et al., 2001).

The postnatal OE has a laminar structure with globose basal cell (GBC) and horizontal basal cell (HBC) progenitors in the basal region, olfactory receptor neurons (ORNs) in the midsection, and sustentacular cells at the apex. In the underlying lamina propria, olfactory ensheathing glia wrap around ORN axon bundles as they target the olfactory bulb in the CNS (Getchell et al., 1984; Farbman, 1992). Bowman's glands in the lamina propria produce mucous delivered through ducts that extend through the OE to the outer surface (Getchell et al., 1984; Farbman, 1992). In contrast, the embryonic OE lacks laminar structure and comprises mostly proliferating progenitors. Several cell types, such as HBCs and sustentacular cells, do not emerge until late embryonic or early postnatal development, with ORN numbers gradually

increasing as embryonic development proceeds. However, the spatiotemporal contributions of embryonic olfactory progenitors to the postnatal OE are primarily unknown.

Although limited, studies using transgenic mice to genetically fate map embryonic progenitor descendants have helped to formulate our current understanding of embryonic OE lineage contributions, which have uncovered either neuron-restricted or glia-restricted embryonic progenitors. Labeled ORNs are detected throughout the OE of FoxG1-cre/reporter mice (Duggan et al., 2008), but regionally restricted to the dorsomedial OE in Nestin-cre/reporter mice (Murdoch and Roskams, 2008), whereas BLBP-cre/reporter mice label only olfactory ensheathing glia (Murdoch and Roskams, 2007). Markers of embryonic precursors with the capacity to produce postnatal neurons together with glia, or additional non-neuronal cells such as sustentacular cells, have not been identified.

In numerous tissues during embryonic development, mammalian Pax genes, transcription factors of the paired domain family, contribute to the regulation of cell proliferation, lineage specification, differentiation, migration, and survival (Lang et al., 2007; Blake et al., 2008). Pax genes also play a role in the development of the OE (Davis and Reed, 1996; LaMantia et al., 2000). For example, although Pax7 mutants have no obvious olfactory abnormality (Mansouri et al., 1996), Pax7 is expressed at early embryonic stages (Jostes et al., 1991; Stoykova and Gruss, 1994) in regions associated with Sox2⁺ putative OE stem cells (LaMantia et al., 2000; Beites et al., 2005; Kawachi et al., 2005; Chen et al., 2009), whose lineage contributions are unknown.

Here we investigate the expression of Pax7 before and during OE ontogeny and use Cre-loxP technology to lineage trace Pax7 progeny to investigate the contributions made by Pax7-expressing embryonic progenitors. Our results reveal novel spatiotemporal patterning of the postnatal OE and identify, for the

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first time, embryonic precursors expressing Pax7 that generate multiple nervous system and chemosensory lineages, including CNS, vomeronasal and olfactory neurons, olfactory glia, and non-neuronal cells.

Materials and Methods

Tissue preparation. Adult and postnatal mice were killed in a CO₂ chamber, perfused with cold PBS and 4% paraformaldehyde (PFA) in PBS, and postfixed in 4% PFA at 4°C (Murdoch and Roskams, 2008). Embryos were immersion fixed in 4% PFA overnight. The day of vaginal plug was defined as embryonic day 0.5 (E0.5). Tissues were cryoprotected in sucrose, embedded in Tissue-Tek medium (OCT; Sakura Finetek), and frozen in liquid nitrogen. Twelve-micrometer sections were stored at –20°C for subsequent analysis.

Immunohistochemistry. Sections were immersed in PBS, permeabilized in 0.1% Triton X-100/PBS, and blocked with 4% normal serum before primary antibody incubation. Secondary antibodies (1:200) used were of specific isotypes conjugated to biotin (Vector Laboratories) or Alexa 568 or Alexa 488 (Invitrogen). Before blocking, Sus4 and transcription factor detection was enhanced by a 15–60 s incubation of the sections with 0.12% trypsin/EDTA (Invitrogen) (longer duration for older ages), followed by washing in PBS. Primary antibodies used were as follows: mouse anti-rat β III tubulin (neuron-specific tubulin-TUJ1; 1:500; Covance); mouse anti-mammalian achaete-scute homolog 1 (Mash1; 1:100; BD Pharmingen); mouse anti-Pax7 and -Pax6 (Developmental Studies Hybridoma Bank; developed by A. Kawakami and C. P. Ordahl); rabbit anti-green fluorescent protein [GFP; 1:400; to detect yellow fluorescent protein (YFP); Millipore]; and Sox2 (1:300; R & D Systems). Note that all YFP panels show anti-GFP immunofluorescence that was confirmed using non-fluorescent immunoperoxidase (VIP) immunohistochemistry with anti-GFP antibodies (supplemental Fig S2, available at www.jneurosci.org as supplemental material), with the exception of the YFP-containing panels in Figure 2B and supplemental Fig S2C (available at www.jneurosci.org as supplemental material), which show endogenous YFP fluorescence. Gift antibodies were mouse anti-rat Sus4 (1:100) from Dr. J. Schwob (Tufts University, Boston, MA) and goat polyclonal olfactory marker protein (OMP; 1:5000) from C. Greer (Yale University, New Haven, CT). Nuclei were stained with 0.5 μ g/ml diaminopyridine imidazole (DAPI), and sections were coverslipped in Vectashield (Vector Laboratories) for fluorescent antigens or 50% glycerol for VIP. Images were visualized with either a Nikon dissecting scope or a Nikon Eclipse 80i microscope using a SPOT camera (SPOT Diagnostic Instruments) with SPOT software (version 4.5). Confocal images were collected on a Zeiss LSM 510 microscope with ZEN software (version 5) and processed using ImageJ (version 1.43r). All images were compiled using Adobe Photoshop 7.0.

Quantitative real-time PCR. Total RNA was isolated from E11.5 mouse embryos using RNeasy B reagent (Tel-Test). Total RNA was reverse transcribed with the iScript cDNA Synthesis kit (Bio-Rad) according to the manufacturer's instructions. Real-time PCR was performed in triplicate using the iQ SYBR Green Super Mix in a Bio-Rad iQ5. Each reaction contained cDNA template, 1 $\times$ iQ SYBR Green Super Mix, and 300 μ M primers. Each plate was run in triplicate. Conditions for amplification were as follows: 3 min at 95°C, followed by 40 cycles of 10 s at 95°C, 30 s at 55°C, and 30 s at 72°C. Melting-curve analysis was performed from 55°C to 95°C, with 1°C/s transitions. The primers used were as follows: Pax7F, 5'-GCTACAGTACAGCCAGTATG-3'; Pax7R, 5'-GTCACTAAGCATGGGTAGATG-3' (McKinnell et al., 2008); β -actinF, 5'-AAGTGTGACGTTGACATCCG-3'; and β -actinR, 5'-GATCCAC-

Figure 1. Pax7 expression in the mouse OE during ontogeny. **A**, Nonfluorescent immunohistochemistry detected Pax7 expression in the E11.5 OE that is restricted to the most rostral region (arrowhead), with robust Pax7 signal intensity in the underlying mesenchymal cells of the lateral nasal process (LNP) (arrow; low magnification in inset). **B**, Pax7 expression was not detected in the E15.5 OE but was found in a few cells at the interface of the OE and underlying mesenchyme (arrows). **C**, **D**, These Pax7+ cells persist in the lamina propria (LP) of the P5 OE (**C**; arrows) but not the adult OE (**D**; arrowhead). The dashed line indicates the basal lamina. Scale bars: **A**, 50 μ m; **B–D**, 100 μ m.

ATCTGCTGGAAGG-3'. Cycle threshold values and amplification curves were obtained using iQ5 Optical System software (version 2.0; Bio-Rad). All data were normalized to β -actin. Relative Pax7 expression was calibrated against wild-type controls where ΔCt_1 = wild-type Ct – Pax7 cre or Pax7 null Ct; ΔCt_2 = wild-type Ct – β -actin Ct; relative Pax7 expression was calculated as $2^{(\Delta Ct_1 - \Delta Ct_2)}$.

Transgenic mice. All experimental procedures were performed in accordance with the Yale Animal Resources Center and Institutional Animal Care and Use Committee policies. Pax7-cre mice (C57BL/6) obtained from M. Capecchi (University of Utah, Salt Lake City, UT) (Keller et al., 2004) were crossed with C57BL/6 female *Gt(Rosa)26Sor^{tm(EYFP)}Cos* mice (a gift from D. Krause, Yale University, New Haven, CT) expressing enhanced YFP from the ROSA26 locus (termed ROSA YFP) (Srinivas et al., 2001). *Gtrosa26^{tm1Sor}* (expressing β -galactosidase protein from the ROSA26 locus) (Soriano, 1999) produced a similar reporter expression pattern after Pax7-cre-mediated excision. Pax7-cre/ROSA YFP double-transgenic mice, where Cre recombinase expression is under the control of the endogenous Pax7 regulatory elements, express YFP without disrupting Pax7 function (Keller et al., 2004). Pax7 LacZ mutant mice were obtained from A. Mansouri (University of Göttingen, Göttingen, Germany) and have been reported previously (Mansouri et al., 1996). Mice were genotyped by PCR for Pax7, Cre (Keller et al., 2004), and YFP. Pax7 primers located within Pax7 exon 10 (5'-GCTCTGGATACACCTGAGTCT-3', 5'-TCGGCCTTCTCTAGGTTCT-GCTC-3'; ck118 and ck256, respectively; 465 bp product) were combined with an *IRE5-Cre* primer (5'-GGATAGTGAACAGGGGCAA-3'; ck172; 340 bp product); ROSA YFP primers were Rosa1 (5'-AAAGTCGCTCTGAGTTGTTAT-3') and Rosa3 (5'-GGAGCGGGAGAAATGGATATG-3'; wild-type ROSA locus, 650 bp product); and Rosa3 and YRF (5'-CGACCACTAC-CAGCAGAACA-3'; ROSA26 YFP, 850 bp product). PCR parameters were as follows: 35 cycles of 95°C at 30 s, 58°C at 30 s, 72°C at 60 s. Efficiency of Cre-lox recombination was verified by the specific colocalization of Pax7 with YFP proteins in Pax7-cre/ROSA YFP embryos and the similar reporter expression patterns detected in Pax7-LacZ (Relaix et al., 2004), Pax7-Zs-green (Bosnakovski et al., 2008), and Pax7-cre/*Gtrosa26^{tm1Sor}* (Keller et al., 2004) mice.

For each time point, at least three Pax7-cre/reporter positive embryos, pups, or mice were analyzed. Sections from the rostral, middle, and caudal OE for coronal sections, or lateral to medial for sagittal sections, were sam-

Figure 2. Fate-mapping Pax7⁺ progenitors in the OE of Pax7-cre/ROSA YFP transgenic mice. **A**, The Pax7-cre construct has an encephalomyocarditis internal ribosomal entry site with the Cre coding sequence (IRES-Cre; gray box) inserted into the 3' untranslated region following the exon 10 (black box) stop codon of the Pax7 locus (represented by the black line) (Keller et al., 2004). Pax7-driven Cre expression occurs without disrupting normal Pax7 function. The arrowhead indicates a remaining flippase recognition target site. **B**, In E11.5 Pax7-cre/ROSA YFP whole-mount embryos, endogenous YFP expression (green) is detected in the midbrain, the hindbrain, the entire anteroposterior axis of the neural tube, the dorsal root ganglia (arrows), and the frontonasal region (arrowhead). **C**, Pax7 (red) and YFP can be detected in mesenchymal cells of the lateral nasal process (LNP; arrows) and the developing OE (arrowhead; boxed region magnified in inset) of transverse E11.5 sections. **D–G**, Pax7, together with YFP expression, is detected in the frontonasal mesenchyme (FNM) and olfactory placode/pit (OP) at E10.5 (arrowhead), the first developmental stage in which we recognized olfactory tissue. The boxed region in **D** is magnified in **E–G**. **H–J**, Immunofluorescence on P5 Pax7-cre/ROSA YFP mice show YFP⁺ cells in the OE (arrowhead) and vomeronasal organ (VNO; arrow) of coronal sections. DAPI nuclear stain is blue in **C–E** and **H**. Individual signals are from Pax7 (**G**), YFP (**B**, **F**, **I**), and DAPI (**J**). Double asterisks indicate dorsal recess. Sep, Septum; LP, lamina propria; FB, forebrain. The dashed line indicates the basal lamina. Scale bars: **B**, 1 mm; **C**, **E–G** (in **C**, **E**), 100 μ m; **D**, 200 μ m; **H–J** (in **H**), 500 μ m.

pled and tested for YFP⁺ cells from each mouse. To determine the rostrocaudal YFP expression pattern, every 10th section was sampled from adult (≥ 60 d) Pax7-cre/ROSA YFP mice.

Cell quantitation. For YFP⁺ cell quantitation, at least three sections per animal, from the rostral, middle, and caudal OE or 72 μ m flanking the Pax7-expressing region in the E11.5 OE, were assayed to adequately sample the OE, from each of three animals per developmental stage. Total YFP⁺ cells per section were divided by the length of OE measured, using ImageJ (version 1.43r). Average total cells per millimeter, assessed by DAPI nuclear stain, were calculated after measuring 100 μ m regions of OE from each turbinate visible on the sections, or all of the visible OE for E11.5.

Results

Pax7 expression in the mouse OE during ontogeny

Pax7 protein expression can be detected in mouse embryos at E7.5–E8.5 in the lateral region of the cephalic neural folds, in the caudal neural plate neuroepithelium, and in the cephalic mesenchyme (supplemental Fig S1, available at www.jneurosci.org as supplemental material), in agreement with previous *in situ* expression beginning at E8 (Jostes et al., 1991). Pax7 is more widely expressed in the E9.5 developing hindbrain, forebrain, and frontonasal mesenchyme (supplemental Fig S1, available at www.jneurosci.org as supplemental material) and is reported in the nose at stages E10–E14.5 (Jostes et al., 1991; Lang et al., 2003). A detailed study showed that E10.5 embryos express Pax7 in the lateral frontonasal mesenchyme and lateral margin of the nasal pit (LaMantia et al., 2000), which was confirmed here (see Fig. 2). To better understand the role of Pax7 progenitors during olfactory development, we used immunohistochemistry to determine the changes in Pax7 expression from embryonic to adult development. By E11.5, the mesenchyme of the lateral nasal process robustly expressed Pax7, which in the OE showed a ventral to dorsomedial gradient (highest to lowest) (Fig. 1A). Pax7 was not readily detected in the E15.5 OE but was found in cells closely juxtaposed to the epithelial–mesenchymal border (Fig. 1B). Similarly, the P5 lamina propria, but not the OE, contained Pax7⁺ cells (Fig. 1C). Neither the adult OE nor the lamina propria contained cells with detectable Pax7 expression (three mice sampled) (Fig. 1D). Thus, the cellular location of Pax7⁺ cells and dynamic expression levels during development suggest that Pax7 precursors may contribute to multiple cell types in the mature OE.

Table 1. Quantitation of YFP+ cells in the olfactory epithelium of Pax7-cre/ROSA YFP mice during ontogeny

	Average millimeter of OE analyzed per animal	Average YFP+ cells per animal	Average YFP+ cells per millimeter	Average total cells per millimeter	Percentage of total cells that are YFP+
E11.5 (n = 3)	3.3	335	102.8 ± 9.1*	630 ± 36.9	16.3
P5 (n = 3)	8.0	710	87.9 ± 21.2*	1307 ± 16.6	6.7
Adult (n = 3)	60.4	755	16.2 ± 2.1	1796 ± 43.0	0.9

Cells were counted from three sections per animal, three animals per stage, as outlined in Materials and Methods. Values indicate means (± SEM). The percentage of YFP+ cells was derived from the average YFP+ cells per millimeter divided by the average total cells per millimeter, multiplied by 100. *p < 0.0001 versus adult.

Fate mapping Pax7+ embryonic progenitors in Pax7-cre/ROSA YFP transgenic mice

To test the lineage contributions of Pax7-expressing cells in the OE, we crossed Pax7-cre transgenic mice, where an IRES-Cre cassette was inserted into exon 10 of Pax7 (Fig. 2*A*) (Keller et al., 2004), with *Gt(ROSA)26Sor^{tm(EYFP)Cos}* reporter mice (hereafter termed ROSA YFP) (Srinivas et al., 2001). In Pax7-cre/ROSA YFP double-transgenic mice, Cre recombinase expression is under the control of the endogenous Pax7 regulatory elements without disrupting Pax7 function. Pax7-dependent Cre expression mediates an excision event that removes a floxed stop cassette, thus allowing continued YFP expression from the ROSA locus in Pax7-expressing cells and their progeny even after Pax7 expression ceases. In whole-mount embryos, we detected endogenous YFP expression in the developing midbrain, hindbrain, neural tube, somites, dorsal root ganglia, and frontonasal region (Fig. 2*B*) similar to endogenous Pax7 expression. The YFP reporter expression pattern seen in Pax7-cre/ROSA YFP embryos is consistent with that reported previously using Pax7-LacZ (Relaix et al., 2004), Pax7-ZsGreen (Bosnakovski et al., 2008), and Pax7-cre/ROSA LacZ mice (Keller et al., 2004), where LacZ and ZsGreen identify Pax7-expressing cells and their immediate or lifelong progeny.

Sections from the frontonasal region of E11.5 Pax7-cre/ROSA YFP mice revealed Pax7+ YFP-negative cells and Pax7+YFP+ cells, which likely represent cells that only recently initiated Pax7 expression and display a delay between excision and detectable levels of reporter, respectively. Similar delays in Cre-mediated reporter expression have been reported before (Joyner and Zervas, 2006; Murdoch and Roskams, 2008). Additionally, Pax7-negative YFP+ cells, the progeny of Pax7-expressing precursors, which had excised and ceased to express Pax7, were also detected (Fig. 2*C*). At E10.5, most to all Pax7-expressing cells in the frontonasal mesenchyme and developing OE coexpress YFP (Fig. 2*D–G*), illustrating the faithful expression of the transgene together with Pax7. Multiple controls ensured the fidelity of our YFP detection in Pax7/reporter transgenic mice [littermate controls without either Pax7-cre or ROSA-YFP alleles, nonfluorescent immunohistochemistry methods, and secondary antibody-only controls (supplemental Fig S2, available at www.jneurosci.org as supplemental material)]. Together, this evidence validates the use of Pax7-cre/ROSA YFP transgenic reporter mice to map Pax7 progeny in olfactory lineages.

Figure 3. The Pax7 lineage contributes to vomeronasal sensory neurons. *A–I*, The P5 vomeronasal organ (VNO) contains a subpopulation of YFP+ (green) vomeronasal receptor neurons that can be TUJ1+ (red; *D–F*, arrows), TUJ1 negative (*D–F*, arrowheads), or OMP+ (red; *G–I*, arrows). The boxed region in *A* is magnified in *D–F*. Individual signals are from YFP (*B, E, H*), TUJ1 (*C, F*), or OMP (*I*); DAPI nuclear stain is blue in *A*. Scale bars: *A–C* (in *A*), *D–I* (in *D*), 100 μ m.

To determine the contribution of embryonic Pax7+ progenitors to the postnatal OE, we tested for YFP+ reporter cells in postnatal day 5 (P5) tissue. YFP+ Pax7 progeny are detected in punctate regions of multiple laminae throughout the P5 OE and the vomeronasal organ, a specialized chemosensory organ (Fig. 2*H–J*). In the OE, each P5 animal (*n* = 3 for this and all subsequent quantitation) averaged 710 YFP+ cells, comprising 87.9 ± 21.2 YFP+ cells/mm of 1307 ± 16.6 total cells/mm (6.7%; *p* < 0.0001 vs adult) (Table 1). The head region also included YFP+ cells in the septal cartilage, head mesenchyme, and nasal glands (Fig. 2*H, I*). These results show that embryonic Pax7 precursors contribute to multiple cell lineages that include chemosensory cells.

Subpopulations of chemosensory neurons and sustentacular cells arise via a Pax7 lineage
Using cell-type-specific antigens, we identified the cell types expressing YFP in the P5 vomeronasal organ. Subpopulations of TUJ1+ (β III neuron-specific tubulin) and OMP-positive vome-

Figure 4. Subpopulations of sustentacular cells and ORNs arise via a Pax7 lineage. **A, B**, Coronal sections from P5 Pax7-cre/ROSA YFP mice show YFP+ (green) cells in the OE, throughout the lamina propria (LP) and in cartilage of the septum (Sep). DAPI is blue in **A**. **C**, Diagram showing the laminar and cellular organization of the postnatal OE that includes from apex to basal lamina, sustentacular cells, ORNs, GBCs, and HBCs; olfactory ensheathing cells, Bowman's glands, and ORN axon bundles are beneath the basal lamina in the lamina propria. **D–F**, YFP+ cells are detected in the apical OE in a subpopulation of Sus4+ (red) sustentacular cells (Sus; apical arrow) and below the apex in Sus4-negative cells (arrowhead). In the LP, Sus4+ cells of Bowman's gland (BG; arrow) do not appear to express YFP, even though YFP+ cells are detected throughout. Cells of Bowman's ducts that emerge into the OE (asterisk), can express YFP. **G–I**, A subpopulation of ORNs (TUJ1+; red) express YFP (arrows). Double asterisks indicate dorsal recess. Scale bars: **A, B** (in **A**), **D–I** (in **D**), 100 μ m. Ax, axon bundles.

ronasal receptor neurons expressed YFP in their cell bodies, dendrites, and axons (Fig. 3*A–I*). YFP+ vomeronasal neurons were seen throughout the apical–basal axis, indicative of both V1R and V2R receptor neurons, respectively (Dulac, 2000).

Within the OE and the underlying lamina propria, multiple cells expressed YFP (Fig. 4*A, B*), whose location and morphology (summarized in Fig. 4*C*) were combined with candidate antigens in an effort to provide specific identity. The Sus4 antigen identifies sustentacular cells and Bowman's duct cells in the OE and Bowman's glands in the lamina propria (Goldstein and Schwob, 1996). With their cell bodies in the apical OE and processes spanning the height of the OE, P5 sustentacular cells represent 251 ± 2.9 Sus4+ cells/mm, of which 4.9 ± 0.7 cells/mm (1.9%) are YFP+ (Fig. 4*D–F*; Table 2). Sus4+ cells found in Bowman's glands did not appear to express YFP (29 glands, three animals), but $10.4 \pm 1.9\%$ of the 46 ducts examined contained YFP+ duct cells (Fig. 4*D–F*). Coexpression of YFP with cell-type-specific antigens, including Sus4, was confirmed by confocal microscopy (Fig. S3, available at www.jneurosci.org as supplemental material).

In the middle OE layers, the cell bodies of TUJ1+ immature ORNs express YFP, as do their axons, dendrites, and dendritic knobs (Fig. 4*G–I*). YFP+ mature OMP+ ORNs were detected in P5 (data not shown) and adult animals (Fig. S4, available at

www.jneurosci.org as supplemental material). In P5 mice, of the 944 ± 16.8 total ORNs/mm, 80.6 ± 6.2 (8.5%) express YFP ($p < 2 \times 10^{-5}$ vs adult) (Table 2). Approximately 3% of basal cells (3.7 ± 0.7 YFP+ cells/mm from a total of 112 ± 1.1 basal cells/mm) express YFP ($p < 0.02$ vs adult) (Table 2).

The adult OE averaged 755 YFP+ cells (Table 1) with 16.2 ± 2.1 YFP+ cells/mm and 1796 ± 43.0 total cells/mm (0.9%) (Table 1). YFP was expressed in $\sim 1\%$ of total sustentacular cells and ORNs and in 0.004% of basal cells (Table 2). Within the YFP population of both adult and P5 animals, ORNs, sustentacular cells, and basal cells had the highest to lowest distribution of YFP+ cells, respectively (Table 3).

A more than twofold increase of YFP+ cells was consistently detected unilaterally compared with contralaterally (but not on the same side in all animals; $n = 3$ per time point), in the P5 (121.8 ± 34.5 vs 54.2 ± 16.2 ; $p < 0.05$) and adult (16.9 ± 0.2 vs 8.2 ± 2.0 ; $p < 0.05$) OE, indicating a bilateral asymmetry in Pax7-derived progeny.

To test whether the insertion of the Cre recombinase gene into the Pax7 locus could modify Pax7 expression and potentially account for the nonuniform pattern of YFP+ cells in the OE, we compared the level of Pax7 expression in transgenic embryos homozygous for Pax7-cre with nontransgenic wild-type embryos (three embryos per genotype). Quantitative reverse transcription-PCR showed no significant difference in the relative expression of Pax7 in wild-type compared with

Pax7-cre homozygous embryos but significant differences in both compared with Pax7 null controls ($p < 0.001$) (supplemental Fig. S5, available at www.jneurosci.org as supplemental material). Likewise, neither the OE ($85.5 \pm 1.8\%$ vs $87.3 \pm 1.5\%$) nor the frontonasal mesenchyme ($98.7 \pm 0.7\%$ vs $98.1 \pm 1.0\%$) revealed any significant differences ($p > 0.05$) in the percentage of Pax7+ cells in Pax7-cre homozygotes compared with wild-type controls, respectively (supplemental Fig. S5, available at www.jneurosci.org as supplemental material). These analyses show that Pax7 expression is stable after Cre insertion and suggest that Pax7 instability cannot account for the unique expression patterns detected in olfactory YFP+ cells. Our results demonstrate that Pax7 embryonic progenitors contribute diverse cell types in unique patterns to the perinatal and adult OE and vomeronasal organ.

Pax7 precursors produce olfactory ensheathing glia

The postnatal lamina propria contains multiple cell types including olfactory ensheathing glia, mesenchymal cells, connective tissues, blood vessels, and fibroblasts. Of particular interest because of their possible use in therapies of spinal cord injury (for review, see Raisman and Li, 2007; Richter and Roskams, 2008; Kocsis et al., 2009) are the olfactory ensheathing glia. Olfactory ensheathing glia are a special type of glial cell that supports the growth of olfactory axons from the peripheral OE to their CNS target, the

olfactory bulb, where they form the nerve fiber layer. Identified by their morphology and location, immediately adjacent to and surrounding TUJ1+ axon bundles in the lamina propria, YFP+ OECs were found in P5 and adult Pax7^{cre}/ROSA YFP mice (Fig. 5*A–D*; supplemental Fig S3, available at www.jneurosci.org as supplemental material), where $94.5 \pm 0.5\%$ of adult OECs are YFP+ (1163 OECs counted). A strong YFP signal is also evident in the nerve fiber layer surrounding the olfactory bulb in the CNS (Fig. 5*E, F*). These results demonstrate that Pax7 derivatives contribute to various cell types in the lamina propria, which include olfactory ensheathing glia found both in the peripheral and central nervous systems.

The Pax7 lineage includes subsets of Pax6-, Sox2-, Mash1-, and intercellular adhesion molecule-1-expressing postnatal olfactory progenitors

To more precisely identify cells arising via a Pax7 lineage and further elucidate their cellular diversity, we assessed the expression of cell-type-specific transcription factors and cell surface molecules in P5 Pax7cre/ROSA YFP mice together with YFP. The Pax6 transcription factor is required for OE development and is expressed postnatally in basal cells, sustentacular cells, and Bowman's gland/duct cells (Davis and Reed, 1996). Sox2, an Sry-related HMG (high mobility group) box transcription factor, is expressed in apical and basal cells in the embryonic (Kawauchi et al., 2004; Beites et al., 2005) and postnatal OE. In P5 Pax7cre/ROSA YFP animals, YFP together with either Sox2 or Pax6 OE expression are detected in a subpopulation of sustentacular and basal cells (Fig. 6*A–H*). In the lamina propria, YFP+ cells closely juxtapose Sox2+ cells of Bowman's glands (Fig. 6*F*).

To test whether GBC or HBC progenitors could descend from Pax7 precursors, we monitored the expression of the basic helix-loop-helix transcription factor Mash1 (Gordon et al., 1995; Cau et al., 1997; Shou et al., 1999) and the intercellular adhesion molecule-1 (ICAM-1) (Carter et al., 2004), respectively. Mash1⁺ cells are detected in the basal layers in addition to the neuronal and sustentacular cell layers of the P5 Pax7cre/ROSA YFP OE (Fig. 6I–L), subsets of which coexpress YFP (Fig. 6J–L). Most ICAM-1⁺ HBCs do not coexpress YFP, but rare ICAM-1⁺YFP⁺ cells could be detected in regions devoid of YFP⁺ ORNs and sustentacular cells (Fig. 6M–O). These results show that subsets of multiple postnatal olfactory progenitors descend from Pax7⁺ precursors.

Subpopulations of embryonic olfactory progenitors arise via the Pax7 lineage

Expression of YFP was used to test for the temporal contributions of YFP+ cells before and during olfactory pit development. Rare YFP+ cells were detected in the cephalic mesenchyme and lateral neural folds of E8.5 Pax7cre-ROSA YFP embryos, whereas more abundant contributions to the hindbrain, forebrain, and frontonasal region were first seen by E9.5 that persisted to E10.5 (supplemental Fig S6, available at www.jneurosci.org as supplemental material). Several cells in the E10.5 frontonasal mesenchyme were Pax7+YFP+, as were cells in the olfactory pit, the only

Table 2. Contribution of YFP+ cells to total cells as a function of cell subtype

	YFP + cells/mm	Total cells/mm	Percentage of YFP+ cells (YFP+ per total cells/mm) * 100
P5 cell type (<i>n</i> = 3)			
Sus	4.9 ± 0.7	251 ± 2.9	1.9
ORN	80.6 ± 6.2	944 ± 16.8	8.5*
BC	3.7 ± 0.7	112 ± 1.1	3.3**
Adult cell type (<i>n</i> = 3)			
Sus	2.3 ± 0.3	256 ± 6.7	0.9
ORN	13.7 ± 1.6	1317 ± 39.3	1.0
BC	0.009 ± 0.002	223 ± 4.3	0.004

Cells were counted from rostral, middle, and caudal sections of OE (3 animals per stage). Values represent means $\pm$ SEM. Sus, Sustentacular cell; ORN, olfactory receptor neuron; BC, basal cell. * $p < 2 \times 10^{-5}$; ** $p < 0.02$. PS versus adult.

Table 3. Contribution of YFP+ cells to each cell subtype as a function of total YFP+ cells in the olfactory epithelium of Pax7-cre/ROSA YFP mice

	Cell subtype distribution of YFP+ cells		
	Basal cells	Olfactory neurons	Sustentacular cells
E11.5 (<i>n</i> = 3)	nd	43%	nd
P5 (<i>n</i> = 3)	4.2 ± 0.8%	91.7 ± 1.3%	5.5 ± 0.5%
Adult (<i>n</i> = 3)	0.68 ± 0.2%	84.1 ± 0.6%	15.2 ± 0.9%

Cell counts were derived from three sections per animal, three animals per developmental stage. Values indicate means ($\pm$ SEM). The percentage of YFP+ cells for each subtype was calculated by dividing the average number of YFP+ cells for each subtype by the total number of YFP+ cells, multiplied by 100. nd, Not done because the antigens used to identify these cell types are not expressed at this developmental stage. * $p < 0.0001$ versus adult.

Figure 5. The Pax7 lineage includes olfactory ensheathing cells. **A–D**, In the lamina propria (LP), TUJ1+ (red) ORN axon bundles (Ax) are surrounded by YFP+ olfactory ensheathing cells that arise via a Pax7 lineage (arrows). **E, F**, YFP+ (maroon, VIP immunohistochemistry) olfactory ensheathing cells continue to ensheath ORN axons into the nerve fiber layer (NFL) of the olfactory bulb (OB; arrow). The boxed region in **E** is magnified in **F**. In addition to olfactory ensheathing cells, YFP+ cells detected in the LP also include presumptive mesenchymal cells, connective tissues, blood vessels, and/or fibroblasts. Individual signals from YFP (**B, D–F**) and TUJ1 (**C**). Sep, Septum. The dashed line indicates the basal lamina. Scale bars: **A–C** (in **C**), 100 μ m; **D**, 50 μ m; **E**, 500 μ m; **F**, 100 μ m.

region at this developmental stage known to contribute to the OE proper (Fig. 2*D–F*).

At E11.5, of the 630 ± 36.9 cells/mm, 102.8 ± 9.1 cells/mm express YFP (16.3%; $p < 0.0001$ vs adult) (Table 1); 43% of YFP⁺ cells are immature TUJ1⁺ neurons (Table 3), suggesting additional contributions from embryonic progenitors. However, only 35% of TUJ1⁺ ORNs (343 of 972 counted) throughout the developing E11.5 OE express YFP (Fig. 7A–D), some of which likely arise via Mash1⁺ neuronal progenitors (Fig. 7E–H). TUJ1 and Mash1 expression was detected also in the few YFP⁺ cells found in the forebrain (Fig. 7A, E). Abundant YFP⁺ cells were detected in the lateral nasal process, many of which coexpressed the presumptive embryonic olfactory stem cell marker Sox2 (Kawauchi

Figure 6. The Pax7 lineage includes Pax6, Sox2, Mash1, and ICAM-1 postnatal olfactory progenitors. **A–C, E–G, I–K.** Coronal sections of P5 Pax7cre/ROSA YFP mice shows subpopulations of Pax6-positive (**A–C**), Sox2-positive (**E–G**), and Mash1-positive (**I–K**) progenitors (all red) that are YFP+ (green). **B–D.** YFP+ cells are seen in a subpopulation of Pax6+ sustentacular cells in the apical layer (arrow) and in Pax6+ progenitors in the basal layers (arrowhead and inset). YFP+ cells in the lamina propria (LP) are closely juxtaposed to Pax6+ YFP-negative cells (asterisk). **F–H.** In the apical OE, YFP+ cells can be detected in a subpopulation of Sox2+ cells (arrow), but less frequently in basal progenitors (arrowhead). Sox2+ cells of Bowman's glands (BG) in the LP are closely juxtaposed by YFP+ cells (**F**; asterisk). **J–L.** YFP is more commonly detected in Mash1+ progenitors (arrowhead) in the middle ORN layer than in the apical or basal layers (arrows). **M–O.** Most ICAM-1+ HBCs do not express YFP (arrowhead), but rare ICAM-1+ YFP+ HBCs can be detected in regions where most other cells do not express YFP (inset magnifies boxed region). Boxes indicate regions highlighted in panels showing individual signals from Pax6 (**C**), Sox2 (**G**), Mash1 (**K**), ICAM-1 (**O**), and YFP (**D, H, L, N**). The dashed line indicates the basal lamina. Scale bars: **A, E, I** (in **A**) and **B–D, F–H, J–O** (in **B**), 100 μ m; **M–O**, insets (in **N**), 50 μ m.

et al., 2004; Beites et al., 2005) (Fig. 7I–L). In general, those progenitors most highly expressing Pax6 did not express YFP, producing a distinct border between YFP-expressing and highly expressing Pax6+ cells (Fig. 7M–P). These patterns display a regional restriction and suggest an early embryonic commitment to the Pax7 lineage in multiple embryonic olfactory progenitors, a small proportion of CNS neurons, and neuronal progenitors.

Discussion

The identity and spatiotemporal location of embryonic OE progenitors, as well as their contributions to the postnatal OE, are primarily unknown. Here, we identified Pax7-expressing cells in the early embryonic OE, which stop expressing Pax7 around E15.5 (Fig. 1). The progeny of embryonic cells expressing Pax7 were genetically fate mapped, leading to the identification of various Pax7 derivatives including olfactory progenitors, non-neuronal cells, CNS, olfactory and vomeronasal neurons, and glia

(Figs. 2–7). These results exemplify the heterogeneity of derivatives generated by embryonic Pax7 precursors.

One limitation of the standard Cre-loxP fate-mapping approach is the inability to spatiotemporally label Pax7 progenitors, hence the precise developmental time point from which Pax7 precursors first emerge and their temporal lineage allocations remain unknown. Before olfactory placode development (before E10.5), we detected YFP+ cells in the anterior neural folds and frontonasal mesenchyme. In the chick embryo, portions of the anterior neural folds are thought to generate the OE (Couly and Le Douarin, 1985; Bhattacharyya and Bronner-Fraser, 2008); however, the frontonasal mesenchyme has no known cellular contribution to the OE. Coincident expression of Pax7 with the YFP reporter in the E10.5 olfactory placode, which subsequently forms the OE, suggests that labeled cells detected within the OE arose via a Pax7+ embryonic olfactory progenitor. The precise origin of olfactory ensheathing cells, however, is currently debated (discussed below).

Pax7 embryonic progenitors compared with postnatal basal cell progenitors

Lineage tracing of GBCs (Goldstein et al., 1998; Huard et al., 1998; Chen et al., 2004) and HBCs (Leung et al., 2007; Iwai et al., 2008) has shown that the most potent clones generate HBCs, GBCs, ORNs, sustentacular cells, and Bowman's duct/gland cells. HBCs can also produce olfactory ensheathing glia *in vitro* (Carter et al., 2004). However, neither GBCs nor HBCs have demonstrated the *in vivo* production of olfactory ensheathing cells. Thus, compared with postnatal basal progenitors, Pax7+ embryonic progenitors share some common cellular descendants but are unique in their ability to additionally produce olfactory ensheathing glia and CNS neurons.

These analyses suggest a possible switch to more restricted progenitors in the OE with aging.

Pax7 embryonic progenitors compared with embryonic olfactory progenitors

Transgenic mice used to fate map embryonic olfactory progenitors identified either glia-restricted (Murdoch and Roskams, 2007) or neuron-restricted (Duggan et al., 2008; Murdoch and Roskams, 2008) embryonic progenitors but not progenitors that contribute to non-neuronal cells, neurons, and glia, such as Pax7 descendants (Figs. 2, 4–6). Additionally, Pax7 neuronal and sustentacular cell descendants revealed a unique pattern of YFP+ progeny not seen in the aforementioned lineage tracing studies, which is found in patches throughout the postnatal OE. This pattern could not be attributed to the instability of Pax7 expres-

sion associated with Cre in the Pax7 locus (supplemental Fig S5, available at www.jneurosci.org as supplemental material).

The mosaic pattern of YFP expression could alternatively be attributable to incomplete recombination. However, since Pax7 expression is not altered, Cre recombinase expression is also likely unaltered, and thus full recombination should result. Multiple data support this view. Incomplete recombination would lead to random excision events resulting in variable YFP expression patterns. Contrary to this, the YFP expression pattern is consistent between equivalently staged samples and in an independent transgenic reporter line, R26R, expressing the LacZ gene (data not shown). The overall YFP expression pattern seen in Pax7-cre/ROSA YFP embryos is consistent with that reported previously using several transgenic mouse lines [Pax7-LacZ (Relaix et al., 2004), Pax7-ZsGreen (Bosnakovski et al., 2008), and Pax7-cre/ROSA LacZ mice (Keller et al., 2004)]. Finally, although the signal intensity of Pax7 protein expression in the olfactory placode and OE is much lower compared with the frontonasal mesenchyme, all Pax7+ cells in the olfactory placode appear to express the YFP reporter (Fig. 2). These results do not support an “incomplete recombination” model and suggest that the pattern of YFP expression seen in Pax7-cre/ROSA YFP mice is reflective of complete recombination events that are Pax7 driven. Discrediting these possible technical caveats highlights the surprising nature of the varied cell types and mosaic distribution of Pax7 derivatives, calling for a mechanistic explanation.

Our study exposes an intriguing difference in the proportion of Pax7-derived cells contributed to the OE versus those contributed to olfactory ensheathing cells. Whereas the first set is composed of a modest and declining contribution, the second set seems stable and robust. A possible explanation for such disparate contributions is that the precursors for both OE and olfactory ensheathing cells are the same (Fig. 8*A–D*), and simply one fate is favored, whereas the others are temporally and quantitatively restricted. Alternatively, different Pax7-expressing progenitors could be responsible for OE versus olfactory ensheathing cell derivation (Fig. 8), each displaying different regulation of proliferation, survival, and differentiation.

The mosaic pattern of Pax7 progeny suggests a considerable contribution from Pax7-negative progenitors to the postnatal OE. Indeed, as the OE expands during embryonic to adult stages, there is a 27-fold decrease (16.3% divided by 0.6%) (Table 1) in the percentage of total cells expressing YFP, even though the average number of YFP+ cells does not appreciably increase beyond P5 (Table 1). Decreases in the YFP contributions to the OE are seen in all cell types examined (Table 1). The proportionate decline in Pax7-derived OE

Figure 7. Subpopulations of embryonic olfactory progenitors arise via Pax7 lineage. *A, E, I, M*, YFP (green) is detected as early as E11.5 on sagittal sections from Pax7-cre/ROSA YFP embryos in the frontonasal process (FNP), developing OE, and a few TUJ1+ neurons and Mash1+ neuronal progenitors in the forebrain (FB; asterisks). DAPI nuclear stain is in blue. *B–D*, YFP is detected in a subpopulation of TUJ1+ (red) ORNs (arrowhead) in the OE and developing olfactory nerve (ON; arrow). *E–H, I–L*, YFP can also be detected in subpopulations of Mash1+ (red; *E–H*) and Sox2+ (red; *I–L*) progenitors throughout the epithelium (arrowheads). YFP expression is detected in a cytokinetic Sox2+ progenitor (asterisk). *M–P*, Cells expressing Pax6 (red) are mostly devoid of YFP expression with a distinct boundary formed between the two (*N–P*, arrow). Inset, Ventral expression of YFP beyond that shown in *N*. Boxes indicate regions highlighted in panels showing individual signals from TUJ1 (*C*), Mash1 (*G*), Sox2 (*K*), Pax6 (*O*), and YFP (*D, H, L, P*). The dashed line indicates the basal lamina. Scale bars: *A, E, I, M*, (in *A*), all others (in *B*), 100 μ m.

progeny with aging could be explained by a progressive depletion in Pax7 progenitors. This makes obvious the participation of alternative, yet undescribed Pax7-negative progenitors, which contribute more highly throughout the OE. If these alternative progenitors are restricted to embryonic stages, like Pax7-precursors, they likely possess greater survival and/or proliferative potential in comparison. Another option considers a set of progenitors present during both embryonic and postnatal development that is responsive to cues for embryonic tissue expansion and postnatal maintenance.

How might local expression of Pax7 define a unique progenitor domain? Regional expression and level of transcription factors, as seen in the developing spinal cord (Briscoe et al., 2000; Bel-Vialar et al., 2007) and detected here between Pax7 and Pax6, could form initial progenitor zones responsible for the fine architecture of the OE. The unique transcriptional profiles characteristic of independent progenitors may confer proliferative and/or survival advantages compared with their competitors. Additional analysis of the contribution of Pax6 and other putative progenitors may resolve these seemingly random mosaic patterns into

Figure 8. Potential lineages of embryonic Pax7+ olfactory progenitors. Non-neuronal cells, neurons, and olfactory ensheathing glia, which no longer express Pax7 (**D**), arise via embryonic Pax7+ progenitors. **A–C**, Potential Pax7 olfactory lineages include a multipotent Pax7+ stem cell (**A**) or more restricted Pax7+ precursor (**B**), each with the capacity to clonally produce multiple cell types, or independent lineages through cell type-restricted precursors (**C**), dependent on the cellular potency of the precursor that first expresses Pax7.

more coherent organizational domains. Importantly, our capacity to identify additional OE progenitors and the expression patterns of their progeny is currently limited by a lack of markers at earlier stages.

Pax7+ muscle and neural crest cells compared with embryonic olfactory progenitors

In muscle, Pax7+ satellite cells are stem/progenitor cells that contribute to postnatal muscle growth and regeneration (Charge and Rudnicki, 2004). Like Pax7+ muscle satellite cells (Collins et al., 2005; Kuang et al., 2007), Pax7+ olfactory precursors likely also represent a hierarchical heterogeneous progenitor cell population with mostly committed progenitors, but whose stem cell function remains to be tested.

Neural crest cells are multipotent migratory cells critical for vertebrate development that contribute to multiple tissues including the peripheral nervous system, cardiovascular, craniofacial bone, and cartilage (Kirby et al., 1983; Le Douarin et al., 1998; Chai et al., 2000; Jiang et al., 2000; Baker, 2005; Nakamura et al., 2006). In the chick, Pax7 has been identified as an early marker of neural crest cells that is required for neural crest development (Basch et al., 2006). Accordingly, Pax7 is expressed in neural crest cell precursors in the dorsal neural tube (Jostes et al., 1991; Mansouri et al., 1996; Lang et al., 2003; Basch et al., 2006), and Pax7-cre/ROSA YFP mice label neural crest derivatives (B. Murdoch and M. I. Garcia-Castro, manuscript in preparation). Interestingly, in dorsal root ganglia, we have detected the Pax7 lineage in neurons but not in S100β+ peripheral glia (data not shown), suggesting that subpopulations of peripheral and olfactory sensory neurons share similar transcriptional lineage profiles.

Our data lead one to question whether neural crest cells contribute to some of the OE derivatives identified here, olfactory ensheathing cells in particular. Quail chick transplants of the anterior neural folds, a region with Pax7 expression (Basch et al., 2006; Otto et al., 2006; Khudyakov and Bronner-Fraser, 2009), contribute to the olfactory placodes (Couly and Le Douarin, 1985; Bhattacharyya and Bronner-Fraser, 2008), from which olfactory ensheathing cells are thought to be derived (Farbman,

1992). Evidence from zebrafish, however, suggests that at least some olfactory ensheathing cells could have a neural crest origin (Whitlock, 2004). Little is known about the lineage of olfactory ensheathing cells in the mouse, beyond that they arise via a BLBP+ precursor (Murdoch and Roskams, 2007). The Pax7 progeny detected in the olfactory mucosa of our Pax7-cre/ROSA YFP mice could arise via Pax7-expressing cells of the olfactory placode, from preplacodal cells, or from neural crest cells. Our experimental model cannot resolve this issue, but transgenic mice used to fate map neural crest derivatives, like Wnt1-cre/reporters (Chai et al., 2000; Jiang et al., 2000), offer encouraging alternatives.

Here we identify embryonic Pax7+ progenitors, whose postnatal progeny reveal novel spatiotemporal patterning of the OE and contribute to multiple cell lineages including neurons, glia, and non-neuronal cells. We propose that Pax7 expression thus identifies the first documented population

of embryonic progenitors capable of producing multiple committed olfactory cell types *in vivo*.

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