Cell Mol Neurobiol (2009) 29:1081–1085
DOI 10.1007/s10571-009-9405-3

ORIGINAL PAPER

Chemical Characterization of Pax6-Immunoreactive Periglomerular Neurons in the Mouse Olfactory Bulb

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Received: 19 January 2009 / Accepted: 7 April 2009 / Published online: 28 April 2009

Abstract The Pax6 transcription factor is a key element along brain development in both the visual and olfactory systems. The involvement of Pax6 in neural fate is well documented in the visual system, whereas in the olfactory system, and in particular in the olfactory bulb (OB), its expression during adulthood has only begun to be elucidated. In the OB, the modulation of primary sensory information is first performed by periglomerular cells (PG). A considerable body of information has unveiled the neurochemical heterogeneity of these neurons. Thus it is well known that Pax6 coexists with dopaminergic/GABAergic mouse PG. However, the presence of this transcription factor in other mouse PG subpopulations has not been studied. Here, we analyzed whether Pax6 is expressed in PG containing the calcium-binding proteins neurocalcin and parvalbumin, and the neuropeptide cholecystokinin. Our results show that Pax6 is not expressed by these PG subpopulations, suggesting that it is mainly restricted to GABAergic PG populations. These findings provide new data in the chemical characterization of mouse Pax6-positive PG.

Keywords Calcium-binding proteins · Dopaminergic neurons · Neuronal phenotypes · Olfactory glomeruli · Transcription factor

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Introduction

During brain development, members of the *pax* gene family play an essential role in the morphogenesis and regulation of different encephalic areas (Stoykova and Gruss 1994; Jiménez et al. 2000; Ziman et al. 2001). This conserved family comprises of nine genes (Wang et al. 2008) that encode transcription factors characterized by DNA-binding sequences (Chalepakis et al. 1992; Czerny et al. 1993). These genes have been implicated in neuronal proliferation, brain regionalization, cell differentiation, and neuronal survival (Lang et al. 2007). Homozygotic Pax mutants die during gestation or shortly after birth, pointing the importance of these genes in organogenesis (Fujiwara et al. 1994; Chi and Epstein 2002). Homozygote Pax6 mutants show abnormalities in forebrain patterning and in cell differentiation, hindering successful prenatal development (Stoykova et al. 1996; Mastick et al. 1997). Heterozygous animals lacking one Pax6 allele exhibit failures in brain morphogenesis, in neuronal proliferation, and cell migration (Dellovade et al. 1998; Warren et al. 1999).

During development, Pax6 is expressed throughout the brain (Osumi et al. 2008). During adulthood, it is mainly restricted to the telencephalon, being abundant in the OB, although it is also expressed in other brain regions (Stoykova and Gruss 1994). The function of Pax6 in OB has been related to the specification and differentiation of new interneurons during adulthood (Hack et al. 2005; Kohwi et al. 2005; Roybon et al. 2009), but few studies addressing the neurochemical features of Pax6-positive bulbar neurons have been performed in adult animals.

In the adult OB, Pax6 is present in different types of interneurons (Dellovade et al. 1998) and is abundantly expressed in PG. PG modulate olfactory transmission from the olfactory nerve (ON) to projecting neurons (Kosaka et al.

Table 1 Primary antibodies

Primary antibody	Reference and catalog number	Primary antibody dilution
Rabbit anti-Pax6	Covance, Berkeley, USA (PRB-278P)	1:1.000
Mouse anti-Pax6	University of Nagoya, Japan	1:1.000
Rabbit-neurocalcin	Nakano et al. (1992)	1:3.000
Rabbit anti-parvalbumin (PV-28)	Swant, Bellinzona, Switzerland (PV-28)	1:2.000
Mouse anti-cholecystokinin	Gift of Cure Digestive Diseases Research Center	1:1.000

1998). Recent studies have demonstrated that Pax6 is essential for the formation of granule cells and PG (Hack et al. 2005; Kohwi et al. 2005).

PG are heterogeneous in their neurochemical composition and have been classified into two types (Kosaka and Kosaka 2007). Type 1 comprises of GABAergic/dopaminergic PG, which contact ON axons, whereas type 2 are also GABAergic and contain calbindin (CB) or calretinin (CR), but they do not establish synapses with ON axons (Kosaka and Kosaka 2007).

Our aim in the present work was to analyze the neurochemical composition of Pax6 PG in the mouse OB in an attempt to discern whether they belong to a specific PG subtype. New data about the specific location of Pax6 in the adult OB are provided.

Methods

Five adult male mice (B6EiC3Sn-a) were used. The animals were handled and sacrificed following current animal care rules of EU (86/609/EEC) and Spanish legislation (RD 1201/2005). After anesthesia, the mice were perfused with a fixative solution [4% paraformaldehyde and 0.2% saturated picric acid in 0.1 M phosphate buffer (PB)]. The OBs were removed and postfixed in the same solution for 2 h, rinsed in PB, and cryoprotected with 30% sucrose.

Thirty-μm-thick coronal sections were obtained using a freezing-sliding microtome. They were collected in phosphate-buffered saline (PBS), pH 7.4. The sections were then washed in PBS and processed using a double immunofluorescence protocol to analyze the possible coexistence of Pax6 expression with neurocalcin (NC), parvalbumin (PV) and cholecystokinin (CCK), and treated with 1% $NaBH_4$ in 0.1 M PB for 20 min (Weruaga-Prieto et al. 1996) and rinsed in PB (3 × 10 min). They were then incubated for 1 h in blocking serum (5% goat serum, 1% DMSO, and 0.2% Triton X-100). Primary antibodies (Table 1) were incubated in the same solution for 72 h at 4°C. Then secondary antibodies were applied together for 2 h. Cy3-conjugated secondary antibody (Jackson laboratories, USA) was used to detect Pax6, and the other one was biotinylated (Vector, USA); both diluted 1:500 and 1:250 in PBS, respectively. To detect biotinylated antibody, sections were incubated with Cy2-conjugated streptavidin diluted 1:200 in PBS for 2 h, mounted, and coverslipped with antifading solution. The resulting material was examined with a confocal microscope (Leica TCS SP2. Germany).

To perform quantitative analyses, five equidistant levels throughout the rostrocaudal OB axis were established, as described (Weruaga et al. 2000). Each level was subsequently divided into four quadrants-dorsal, ventral, medial

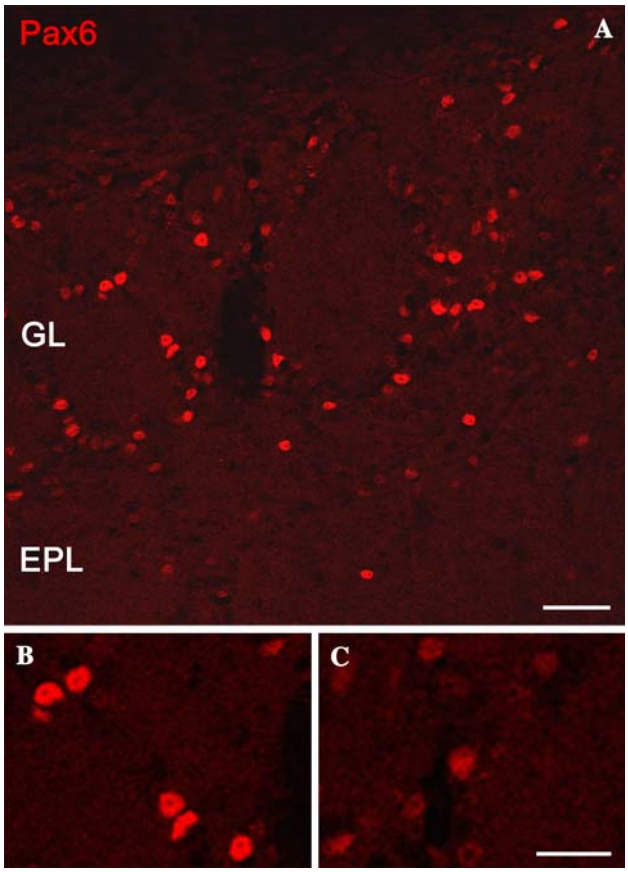

Fig. 1 Distribution pattern of Pax-6 positive neurons in the mouse OB. **a** shows the distribution pattern of Pax6 positive neurons in the mouse GL. They mainly surround the glomeruli. Pax6 staining only reveals cell nuclei, appearing both vividly (**b**) and faintly immunolabeled (**c**). *Scale bars* (A = 40 μm, B, C = 20 μm)

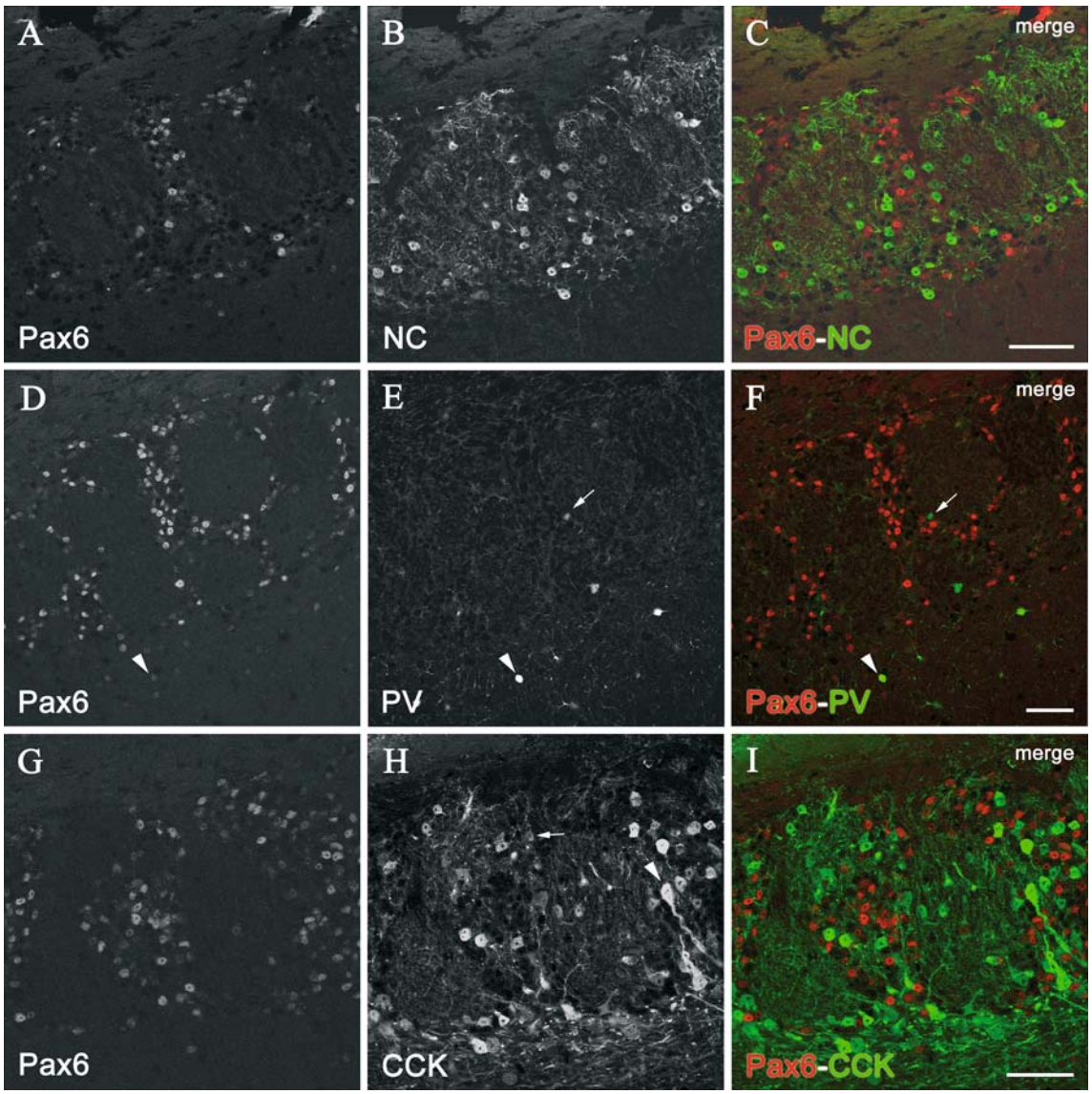

Fig. 2 Pax6 is not expressed by NC-, PV- and CCK-containing mouse PG. **a**, **b**, **c** Co-staining for Pax6 and NC. Mouse Pax6-positive PG (**a**) do not express NC (**b**, **c**). **d**, **e**, **f** Double immunofluorescence for Pax6 and PV shows that the few PV-positive PG do not contain Pax6 (*arrow* in **e**, **f**). By contrast, double-stained neurons can be seen in the EPL (arrowheads). **g**, **h**, **i** Colocalization analysis for Pax6 and CCK. None of the CCK-immunostained populations located in the GL (**h**), nor PG (*arrow*) nor external tufted cells (*arrowhead*) contains Pax6 (**i**). *Scale bars* = 40 μm

and lateral- and focal planes of double-labeled sections were obtained in each quadrant using confocal microscopy. Positive neurons included in these divisions were counted for each combination using the ImageJ software (Scion Corporation, USA). The results are expressed as the percentage of Pax6-positive PG expressing the other markers.

Results

Immunohistochemistry for Pax6 revealed numerous positive neurons in the GL of mouse OB, mainly surrounding the glomeruli (Fig. 1). Previous studies have analyzed whether Pax6 was present in dopaminergic-, GABAergic-calretinin-, or calbindin-positive PG (Hack et al. 2005; Kohwi et al. 2005; Allen et al. 2007). Here, we built upon this using double immunolabeling analysis for Pax6 elements. We employed the markers (NC, PV and CCK) widely used for PG characterization in the rat (Brinón et al. 1999; Gutièrrez-Mecinas et al. 2005) but little used in the mouse.

We observed that only about one percent of the Pax6-positive PG also expressed NC (Fig. 2; 1.3 ± 0.63%), whereas two percent of NC-reactive PG contained NC (2.05 ± 0.93%). PV-immunopositive interneurons were mainly confined to the EPL of the mouse OB, although a

few PV-labeled PG were seen in the GL. Analysis of this small PG subpopulation revealed that no PV-positive PG expressed Pax6, and hence that most belonged to separate classes of PG (Fig. 2). In the GL, CCK-immunostaining revealed both external tufted cells and PG. The combination of CCK and Pax6 revealed that very few CCK-immunopositive PG contained Pax6 (1.37 ± 0.62%; Fig. 2).

Discussion

Neurochemical studies addressing PG have classically been performed in the rat OB (Kosaka et al. 1998; Crespo et al. 2003; Gutièrrez-Mecinas et al. 2005; Baltanás et al. 2007). Nevertheless, recent studies have begun to elucidate the chemical properties of mouse PG (Kosaka and Kosaka 2007; Parrish-Aungst et al. 2007).

This is the first work in which the coexistence of PV/NC/CCK and Pax6 has been investigated. Our results show that neither of these PG subpopulations contains Pax6. Previous studies have reported that neither NC- nor PV-positive mouse PG are GABAergic (Kosaka and Kosaka 2008). In the rat, CCK-reactive PG are also non-GABAergic (Gutièrrez-Mecinas et al. 2005). Assuming that this feature is preserved in mice, Pax6 is therefore not expressed by at least three non-GABAergic mouse PG subpopulations.

Pax6 is expressed by dopaminergic mouse PG and is therefore present in type 1 PG (Hack et al. 2005; Kohwi et al. 2005; Allen et al. 2007). Moreover, it has been demonstrated that other TH-immunoreactive juxtaglomerular neurons also express Pax6 (Hack et al. 2005). These observations agree with the notion that Pax6 is required for the proliferation, specification, and/or maintenance of most dopaminergic/GABAergic populations (Vitalis et al. 2000; Kroll and O'Leary 2005). Indeed, Pax6 mutants exhibit a decrease in dopaminergic PG (Dellovade et al. 1998).

Very few Pax6-positive PG express CB (Dellovade et al. 1998). Interestingly, Pax6 is necessary for CB-positive PG to differentiate (Hack et al. 2005), although this feature is not preserved during adulthood. In fact, CB-positive PG are not affected in Pax6-mutants (Dellovade et al. 1998). Therefore, Pax6 seems to be necessary during the development of CB-positive PG, but not for their functional activity or survival during adulthood (Allen et al. 2007). In contrast to previous reports (Dellovade et al. 1998), one subpopulation of CR-positive PG coexpresses Pax6 (Allen et al. 2007), and these should therefore be included in type 2 PG. However, it has also been demonstrated that the development and acquisition of the CR-phenotype depends on the Sp8 transcription factor (Waclaw et al. 2006). Accordingly, the presence of Pax6 during adulthood in a CR-reactive subpopulation of PG does not seem to be crucial for either the acquisition of their phenotype or their survival. Moreover, this population is not affected in Pax6 mutant mice (Dellovade et al. 1998).

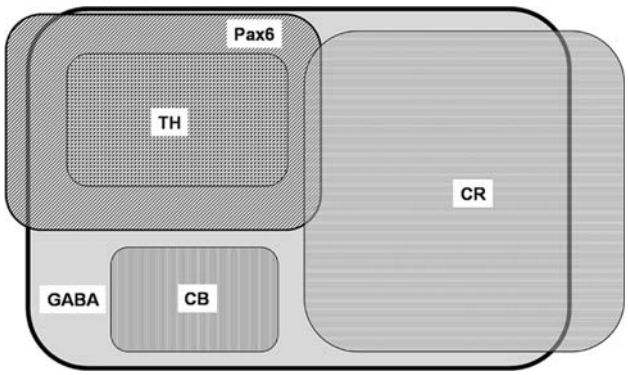

Fig. 3 Schema of colocalization of Pax6-positive PG. The diagram of the coexpression of Pax6-positive PG is based on previously reported results (Dellovade et al. 1998; Hack et al. 2005; Kohwi et al. 2005; Allen et al. 2007; Kosaka and Kosaka 2007). All of the TH-reactive PG contain Pax6, and about 85% of Pax6-reactive PG are GABAergic (Dellovade et al. 1998; Hack et al. 2005; Kohwi et al. 2005; Allen et al. 2007). Additionally, a partial coexpression of Pax6 with CR (15%) has been reported the mouse PG (Allen et al. 2007). None of Pax6-immunopositive mouse PG contains CB (Allen et al. 2007)

Our study shows that in the adult OB Pax6 is specifically expressed in specific PG subpopulations, complementing previous data regarding the chemical features of Pax6-positive mouse PG (Fig. 3). The Pax6 expression observed suggests that it is restricted to GABAergic PG and seems to regulate the survival of the dopaminergic/GABAergic subpopulation.

Acknowledgments The authors would like to thank Dr. Nakano for kindly providing the NC antiserum. The mouse antibody raised against CCK was provided by CURE Digestive Diseases Research Center, Antibody/RIA Core, under NIH grant DK41301. The authors express their gratitude to N. Skinner for revising the English version of the manuscript. This work was supported by the Ministerio de Educación y Ciencia (SAF2006-05705), the Ministerio de Sanidad y Consumo (PNSD 2008/1/013), the Junta de Castilla y León and the Center for Regenerative Medicine and Cell Therapy of Castilla y León.

References

Allen ZJII, Waclaw RR, Colbert MC, Campbell K (2007) Molecular identity of olfactory bulb interneurons: transcriptional codes of periglomerular neuron subtypes. J Mol Histol 38:517–525. doi:10.1007/s10735-007-9115-4

Baltanás FC, Weruaga E, Airado C, Valero J, Recio JS, Díaz D, Alonso JR (2007) Chemical heterogeneity of the periglomerular neurons in the olfactory bulb. A review. Eur J Anat 11:123–147

Briñón JG, Martínez-Guijarro FJ, Bravo IG, Arévalo R, Crespo C, Okazaki K, Hidaka H, Aijón J, Alonso JR (1999) Coexpression of neurocalcin with other calcium-binding proteins in the rat main olfactory bulb. J Comp Neurol 407:404–414. doi:10.1002/(SICI)1096-9861(19990510)407:3<404::AID-CNE8>3.0.CO;2-9

Chalepakis G, Tremblay P, Gruss P (1992) Pax genes, mutants and molecular function. J Cell Sci Suppl 16:61–67

Chi N, Epstein JA (2002) Getting your Pax straight: Pax proteins in development and disease. Trends Genet 18:41–47. doi:10.1016/S0168-9525(01)02594-X

Crespo C, Gracia-Llanes FJ, Blasco-Ibáñez JM, Gutièrrez-Mecinas M, Marqués-Marí AI, Martínez-Guijarro FJ (2003) Nitric oxide synthase-containing periglomerular cells are GABAergic in the rat olfactory bulb. Neurosci Lett 349:151–154. doi:10.1016/S0304-3940(03)00819-X

Czerny T, Schaffner G, Busslinger M (1993) DNA sequence recognition by Pax proteins: bipartite structure of the paired domain and its binding site. Genes Dev 7:2048–2061. doi:10.1101/gad.7.10.2048

Dellovade TL, Pfaff DW, Schwanzel-Fukuda M (1998) Olfactory bulb development is altered in small-eye (*Sey*) mice. J Comp Neurol 402:402–418. doi:10.1002/(SICI)1096-9861(19981221)402:3<402::AID-CNE8>3.0.CO;2-0

Fujiwara M, Uchida T, Osumi-Yamashita N, Eto K (1994) Uchida rat (*rSey*): a new mutant rat with craniofacial abnormalities resembling those of the mouse *Sey* mutant. Differentiation 57:31–38. doi:10.1046/j.1432-0436.1994.5710031.x

Gutièrrez-Mecinas M, Crespo C, Blasco-Ibáñez JM, Gracia-Llanes FJ, Marqués-Marí AI, Martínez-Guijarro FJ (2005) Characterization of somatostatin- and cholecystokinin-immunoreactive periglomerular cells in the rat olfactory bulb. J Comp Neurol 489:467–479. doi:10.1002/cne.20649

Hack MA, Saghatelyan A, de Chevigny A, Pfeifer A, Ashery-Padan R, Lledó PM, Götz M (2005) Neuronal fate determinants of adult olfactory bulb neurogenesis. Nat Neurosci 8:865–872

Jiménez D, García C, de Castro F, Chédotal A, Sotelo C, de Carlos JA, Valverde F, López-Mascaraque L (2000) Evidence for intrinsic development of olfactory structures in Pax-6 mutant mice. J Comp Neurol 428:511–526. doi:10.1002/1096-9861(20001218)428:3<511::AID-CNE8>3.0.CO;2-I

Kohwi M, Osumi N, Rubenstein JLR, Ávarez-Buylla A (2005) Pax6 is required for making specific subpopulations of granule and periglomerular neurons in the olfactory bulb. J Neurosci 25:6997–7003. doi:10.1523/JNEUROSCI.1435-05.2005

Kosaka K, Kosaka T (2007) Chemical properties of type 1 and type 2 periglomerular cells in the mouse olfactory bulb are different from those in the rat olfactory bulb. Brain Res 1167:42–55. doi:10.1016/j.brainres.2007.04.087

Kosaka T, Kosaka K (2008) Heterogeneity of parvalbumin-containing neurons in the mouse main olfactory bulb, with special reference to short-axon cells and betaIV-spectrin positive dendritic segments. Neurosci Res 60:56–72. doi:10.1016/j.neures.2007.09.008

Kosaka K, Toida K, Aika Y, Kosaka T (1998) How simple is the organization of the olfactory glomerulus? the heterogeneity of so-called periglomerular cells. Neurosci Res 30:101–110. doi:10.1016/S0168-0102(98)00002-9

Kroll TT, O'Leary DD (2005) Ventralized dorsal telencephalic progenitors in Pax6 mutant mice generate GABA interneurons of a lateral ganglionic eminence fate. Proc Natl Acad Sci USA 102:7374–7379. doi:10.1073/pnas.0500819102

Lang D, Powell SK, Plummer RS, Young KP, Ruggeri BA (2007) PAX genes: roles in development, pathophysiology, and cancer. Biochem Pharmacol 73:1–14. doi:10.1016/j.bcp.2006.06.024

Mastick GS, Davis NM, Andrew GL, Easter SS Jr (1997) Pax-6 functions in boundary formation and axon guidance in the embryonic mouse forebrain. Development 124:1985–1997

Nakano A, Terasawa M, Watanabe M, Usuda N, Morita T, Hidaka H (1992) Neurocalcin, a novel calcium binding protein with three EF-hand domains, expressed in retinal amacrine cells, ganglion cells. Biochem Biophys Res Commun 186:1207–1211. doi:10.1016/S0006-291X(05)81534-7

Osumi N, Shinohara H, Numayama-Tsuruta K, Maekawa M (2008) Concise review: Pax6 transcription factor contributes to both embryonic and adult neurogenesis as a multifunctional regulator. Stem Cells 26:1663–1672. doi:10.1634/stemcells.2007-0884

Parrish-Aungst S, Shipley MT, Erdelyi F, Szabo G, Püche AC (2007) Quantitative analysis of neuronal diversity in the mouse olfactory bulb. J Comp Neurol 501:825–836. doi:10.1002/cne.21205

Roybon L, Deierborg T, Brundin P, Li JY (2009) Involvement of Ngn2, Tbr and NeuroD proteins during postnatal olfactory bulb neurogenesis. Eur J NeuroSci 29:232–243. doi:10.1111/j.1460-9568.2008.06595.x

Stoykova A, Gruss P (1994) Roles of *Pax*-genes in developing and adult brain as suggested by expression patterns. J Neurosci 14:1395–1412

Stoykova A, Fritsch R, Walther C, Gruss P (1996) Forebrain patterning defects in Small eye mutant mice. Development 122: 3453–3465

Vitalis T, Cases O, Engelkamp D, Verney C, Price DJ (2000) Defect of tyrosine hydroxylase-immunoreactive neurons in the brains of mice lacking the transcription factor Pax6. J Neurosci 20:6501–6516

Waclaw RR, Allen ZJ 2nd, Bell SM, Erdélyi F, Szabó G, Potter SS, Campbell K (2006) The zinc finger transcription factor Sp8 regulates the generation and diversity of olfactory bulb interneurons. Neuron 49:503–516. doi:10.1016/j.neuron.2006.01.018

Wang Q, Fang WH, Krupinski J, Kumar S, Slevin M, Kumar P (2008) Pax genes in embryogenesis and oncogenesis. J Cell Mol Med 12:2281–2294. doi:10.1111/j.1582-4934.2008.00427.x

Warren N, Caric D, Pratt T, Clausen JA, Asavaritikrai P, Mason JO, Hill RE, Price DJ (1999) The transcription factor, Pax6, is required for cell proliferation and differentiation in the developing cerebral cortex. Cereb Cortex 9:627–635. doi:10.1093/cercor/9.6.627

Weruaga E, Velasco A, Briñón JG, Arévalo R, Aijón J, Alonso JR (2000) Expression of neuronal nitric oxide synthase/NADPH-diaphorase during olfactory deafferentation and regeneration. Eur J NeuroSci 12:1177–1193. doi:10.1046/j.1460-9568.2000.00003.x

Weruaga-Prieto E, Eggli P, Celio MR (1996) Rat brain oligodendrocytes do not interact selectively with axons expressing different calcium-binding proteins. Glia 16:117–128. doi:10.1002/(SICI)1098-1136(199602)16:2<117::AID-GLIA4>3.0.CO;2-0

Ziman MR, Rodger J, Chen P, Papadimitriou JM, Dunlop SA, Beazley LD (2001) Pax genes in development and maturation of the vertebrate visual system: implications for optic nerve regeneration. Histol Histopathol 16:239–249