

Expression of Pax2 protein during the formation of the central nervous system in human embryos

A. Namm^{1, 2}, A. Arend¹, M. Aunapuu^{1, 2}

¹Department of Anatomy, University of Tartu, Tartu, Estonia

²Department of Morphology, Estonian University of Life Sciences, Tartu, Estonia

[Received 23 December 2013; Accepted 4 February 2014]

Members of the paired box (Pax) gene family are expressed in distinctive regions of the developing central nervous system, supporting a role of neural patterning. In this study, Pax2 protein expression was examined in the developing neural tube by immunohistochemistry methods in 30 human embryos of Carnegie stages (CS) 10–20 collected after legal abortions. Pax2 expression was detected along the boundaries of main divisions of the developing brain and spinal cord. However, Pax2 expression was found to be stronger in the developing brain than in the spinal cord of the same young embryos in CS 10–14, which was the most remarkable at CS 10. Pax2 expression was detected in the developing forebrain, midbrain and hindbrain. At later stages (CS 16–20) Pax2 expression was observed in the midbrain-hindbrain boundary and also in the developing diencephalon and cerebellum. In the wall of developing spinal cord Pax2 expression was detected in the ventricular, mantel and marginal layers. Pax2 staining was seen to increase throughout the later stages of spinal cord development and significantly stronger expression was found at CS 16–20 compared to CS 10. Furthermore, spatially restricted expression of Pax2 was observed along the compartmental dorsal-ventral axis of the spinal cord as Pax2 staining was weaker in the ventricular layer of the ventral part of the developing spinal cord compared with developing area of dorsal part. (Folia Morphol 2014; 73, 3: 272–278)

Key words: human embryos, Pax2, brain, spinal cord

INTRODUCTION

The family of paired box (Pax) genes constitutes a group of developmental genes with a highly conserved paired domain, which encode nuclear transcription factors [24]. Evidence from mice and human embryos indicates that Pax genes play an important role in early mammalian embryogenesis. Furthermore, the members of the Pax genes family have been shown to play critical roles in the formation of the nervous system as the restricted expression pattern along the craniocaudal and dorsoventral axes of the neural tube during early embryogenesis has demonstrated [21].

The Pax gene family consists of 9 members divided into subgroups based on various aspects of similarity: subgroup I includes Pax1 and Pax9; subgroup II includes Pax2, Pax5 and Pax8; subgroup III includes Pax3 and Pax7; subgroup IV includes Pax4 and Pax6 [21].

Except for Pax1 and Pax9, all Pax genes are spatially and temporally expressed during the development of the central nervous system [24]. At early developmental stages, Pax3, Pax6 and Pax7 are expressed in almost the entire developing neural tube, only Pax6 is excluded from the mesencephalic roof [24]. In later stages, the expression of Pax3/Pax7 is retracted with a rostral

limit at the level of posterior commissure, while Pax6 expression in the forebrain persists during embryogenesis and even in certain structures in adulthood [17]. For the second group of late expressed Pax genes (Pax2, Pax5, Pax8), the midbrain-hindbrain boundary is believed to be the rostral limit [10, 16, 24]. Several animal studies have revealed the importance of Pax2 gene in the establishment and maintenance of the midbrain/hindbrain organiser region, which has been shown to control the development of the midbrain and cerebellum [1, 14, 23].

Despite numerous investigations on Pax genes in animals, only limited results exist about the developmental role of human Pax3 and Pax6 genes [5, 20], while the data about Pax2 genes in the human nervous system formation are very scanty [18, 19]. Therefore, the aim of this study was to determine the temporal and spatial expression of Pax2 protein during the early stages (Carnegie stages [CS] 10–20) of spinal cord and brain development in human embryos. The period from CS 10 (approximately 22–23 days of development) to CS 20 (approximately 51–53 days of development) is critical, as during that time all important changes take place in the size, shape and complexity of the developing brain and spinal cord [11].

MATERIALS AND METHODS

The morphological study was performed on 30 human embryos at CS 10–20. The human embryos were obtained by medical abortions in Tartu University Hospital. The study was approved by the Ethics Review Committee on Human Research of the University of Tartu (no 183/M-40, issued on 29.06.2009). The embryos were fixed in 4% paraformaldehyde and embedded in paraffin according to standard methods. Tissue blocks were cut serially in transversal direction with microtome Ergostar HM-200 (Microm, Germany). The embryos were classified by CS [11] and examined using an Olympus BX-50 microscope. Specimens for light microscopy were stained using the haematoxylin and eosin for general orientation to sections. Slides were observed and photographed by a Zeiss Axiophot 2 microscope (Zeiss, Germany). According to CS, the embryos were grouped as follows: CS 10 — 4 embryos (in total 78 sections per CS 10), CS 12 — 4 embryos (92 sections per CS 12), CS 14 — 10 embryos (154 sections per CS 14), CS 16 — 6 embryos (130 sections per CS 16), CS 18 — 4 embryos (136 sections per CS 18), CS 20 — 2 embryos (73 sections per CS 20).

Immunohistochemistry

Three- μm thick paraffin sections mounted on Super-Frost slides (Menzel-Gläser, Germany) were deparaffinised and rehydrated. Peroxidase activity was blocked by 0.6% H_2O_2 (Fluka, France) in methanol. Sections were washed in PBS (pH = 7.4) and after that in distilled water (3×2 min). The slides were irradiated with 0.001 M citrate buffer (pH = 6.0) for 10 min using a microwave oven (Daewoo, model No KOG-840P) at 98°C. The slides were treated with normal 1.5% goat serum (Gibco, Invitrogen, USA) for 30 min at room temperature and incubated with the primary antibody Pax2 (4 $\mu\text{g}/\text{mL}$ dilution in 0.1 M PBS, abcam, UK) overnight at 4°C in a humidity chamber. The primary antibody used was rabbit polyclonal antibody to Pax2 (abcam, ab23799), which has been reported to perform excellently in immunohistochemical applications with paraffin embedded human samples [13] as well in Western blotting applications with human tissue samples [6].

On the next day, the sections were incubated with the universal secondary antibody (Vectastain ABC Universal Kit, Burlingame, USA) for 30 min at room temperature. Peroxidatic activity was detected with DAB (3,3'-Diaminobenzidine; Vector, USA) and the sections were counterstained with haematoxylin. The labelling was expressed by a subjective scale ranging from 0 to 3 (0 — no staining, 1 — weak staining, 2 — moderate staining, 3 — strong staining; Fig. 1). Immunohistochemistry negative controls were performed by omitting the primary antibody. The evaluation was performed by 2 independent observers in a blinded fashion. The mean of estimations was calculated and used for statistical analysis. The data were analysed by Kruskal-Wallis test (nonparametric ANOVA) with the level of significance set as $p < 0.05$.

RESULTS

The expression of Pax2 protein was detected in the forming neural tube and the developing spinal cord of human embryos at all the studied stages of development. However, variations seem to exist in immunostaining intensity at different developmental stages (Table 1). If Pax2 expression in CS 10–14 was relatively weaker in the developing spinal cord, then stronger expression was found in CS 16–20. Statistically significant weaker expression of Pax2 was found in the embryos of CS 10 as compared to CS 16–20 (Table 1, Fig. 2). In the embryos of CS 16–20, Pax2 was detected in the ventricular (neuroepithelial) layer, mantle layer and also in the marginal layer of the developing spinal cord (Table 1, Figs. 3, 4).

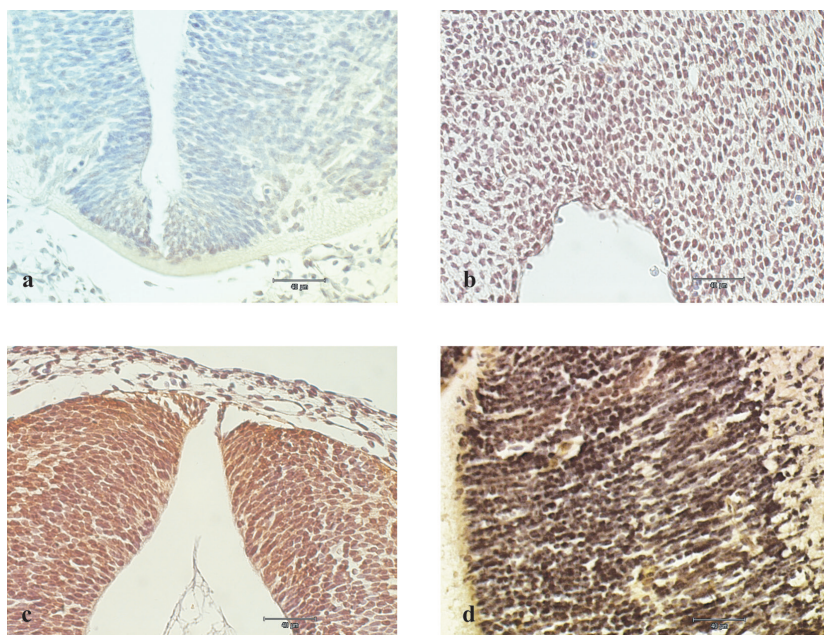


Figure 1. A set of micrographs of the developing human spinal cord illustrating the criteria for evaluation of the staining intensity; a. 0 (no staining); b. 1 (weak staining); c. 2 (moderate staining); d. 3 (strong staining).

Table 1. Expression of Pax2 in the developing spinal cord and brain

| Carnegie stage | 10 | 12 | 14 | 16 | 18 | 20 |
|----------------|--------------------------------|-------------|-------------|--------------------------|--------------------------|--------------------------|
| Spinal cord | 1.37 ± 0.09 ^{a,b,c,d} | 1.61 ± 0.07 | 1.56 ± 0.07 | 1.80 ± 0.14 ^a | 1.83 ± 0.06 ^b | 1.80 ± 0.05 ^c |
| Brain | 2.0 ± 0.05 ^d | 1.91 ± 0.09 | 1.87 ± 0.06 | 1.87 ± 0.09 | 1.89 ± 0.08 | 1.83 ± 0.07 |

Immunostaining intensity was graded as 0 (no staining), 1 (weak staining), 2 (moderate staining) or 3 (strong staining); the data are presented as mean ± SEM. Significant differences ($p < 0.01$) in staining intensity between groups are indicated with the same letters; a — Pax2 expression of the developing spinal cord in CS10 vs. Pax2 expression in the developing spinal cord in CS16; b — Pax2 expression of the developing spinal cord in CS10 vs. Pax2 expression in the developing spinal cord in CS18; c — Pax2 expression of the developing spinal cord in CS10 vs. Pax2 expression in the developing spinal cord in CS20; d — Pax2 expression of the developing spinal cord in CS10 vs. Pax2 expression in the developing brain in CS10

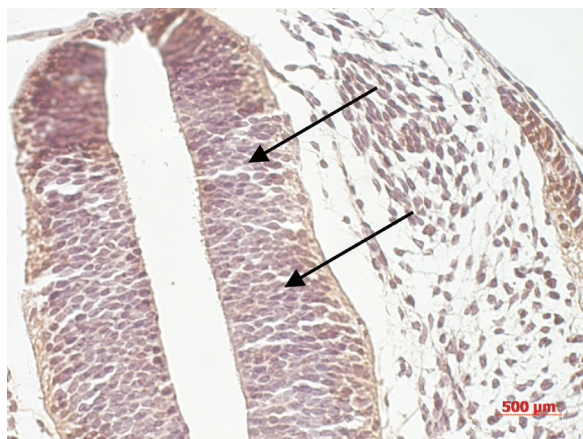


Figure 2. Transverse section of the spinal cord of the human embryo at CS 12. The arrows indicated the ventricular layer of the developing neural tube, where weak expression of Pax2 was detected.

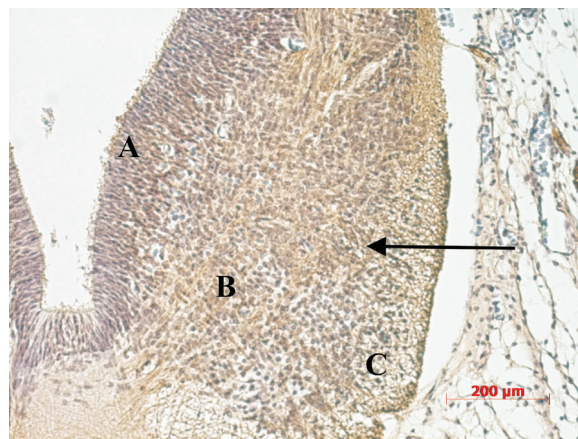


Figure 3. Transverse sections of the spinal cord of human embryo at CS 18; A — ventricular layer; B — mantle layer; C — marginal layer, arrow indicates the anterior horn. Expression of Pax2 in the ventricular, mantle and marginal layers.

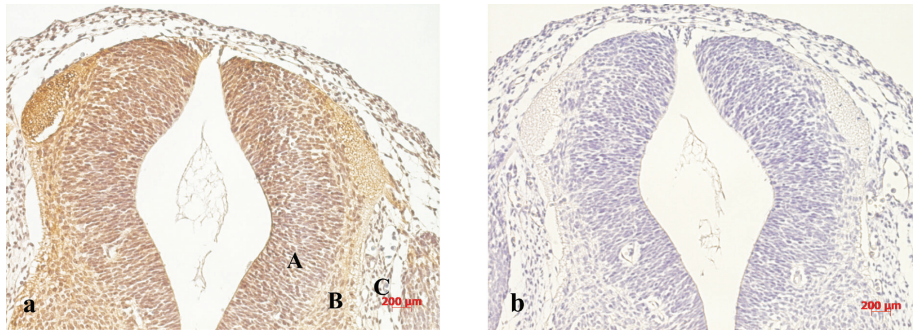


Figure 4. Transverse sections of the spinal cord of human embryo at CS 16; A — ventricular layer; B — mantle layer; C — marginal layer; a. Expression of Pax2 in the ventricular, mantle and marginal layer; b. Negative control, where during processing primary antibody was omitted.

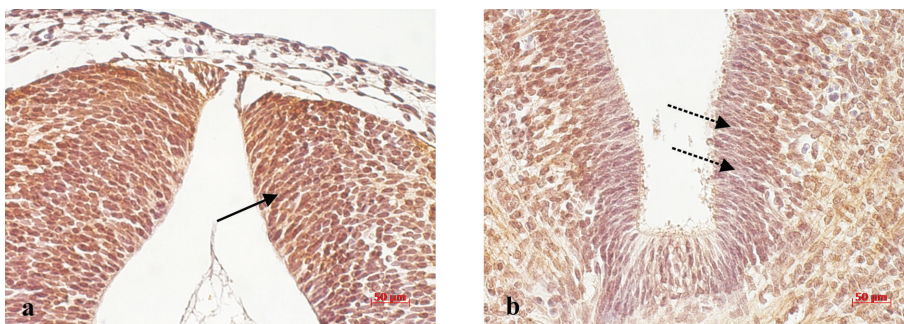


Figure 5. Transverse sections of the spinal cord of the human embryos at CS 18; a. Expression of Pax2 in ventricular layer of the dorsal part of the developing spinal cord (arrow); b. Expression of Pax2 in the ventral part of the developing spinal cord. Double arrows indicated the weaker expression of Pax2 in the ventricular layer.

Comparing Pax2 expression in the dorsal and ventral parts of the developing spinal cord, varying staining intensity was seen in the embryos of CS 14–20. Significant expression of Pax2 in outer layers of the wall of the developing spinal cord on both dorsal and ventral parts was noted, but in the ventricular layer, the expression of Pax2 was more extensive in the dorsal part than in the ventral part of the spinal cord (Fig. 5a, b).

Comparing the expression of Pax2 in the developing spinal cord and brain, more intensive immunostaining was noted in the developing brain at early stages of development, and statistically significant difference was found at CS 10 (Table 1). Concerning the expression of Pax2 in the developing brain, staining was seen in the wall of 3 primary brain vesicles: in the forebrain, midbrain and hindbrain (Figs. 6a–c). Expression of Pax2 was also detected at the junction of the caudal midbrain and the rostral hindbrain in the developing brain — strong Pax2 staining was seen in the region of the isthmus at later stages, CS 16–20 (Fig. 7). Expression of Pax2 was found also

in the developing diencephalon: staining was noted in the outer regions of the dorsal (alar) plates and, as a thin strip, in the ventral part of the diencephalon (Fig. 8). Furthermore, Pax2 expression was detected in the ventricular, mantle and marginal layers of the developing cerebellum in the embryos of CS 18–20 (Fig. 8b).

DISCUSSION

In this study, Pax2 protein immunostaining was analysed in the developing nervous system in human embryos at CS 10–20. The results of the study demonstrated a spatially and temporally restricted expression pattern of Pax2 in the developing spinal cord and brain.

In the early stages of development (CS 10–14), the forming spinal cord and brain vesicles consist of neuroepithelial cells, which extend over the entire thickness of the wall and divide rapidly to constitute the ventricular (neuroepithelial) layer [9, 11]. In our study, moderate PAX2 immunostaining was noted in the wall of the forming spinal cord during these early stages (Table 1). Throughout the following stages,

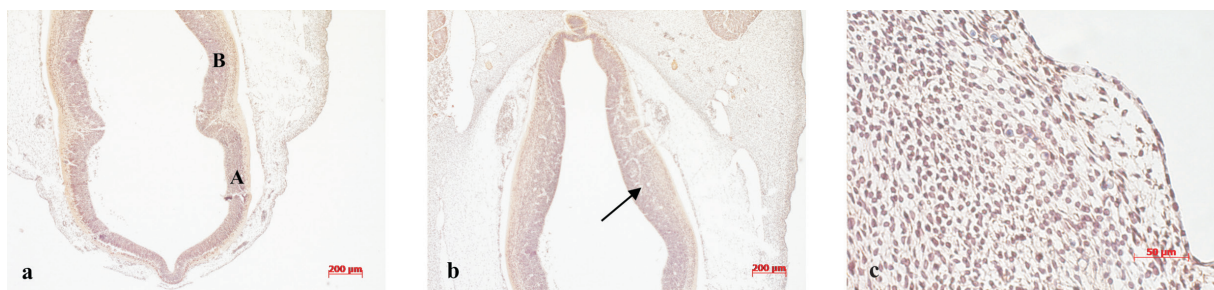


Figure 6. Transverse section of the brain of human embryos at CS 12; **a.** Expression of Pax2 in the wall of the hindbrain (A) and midbrain (B); **b.** Expression of Pax2 in the wall of the forebrain (arrow); **c.** Higher magnification of the wall of the forebrain from panel **b.**

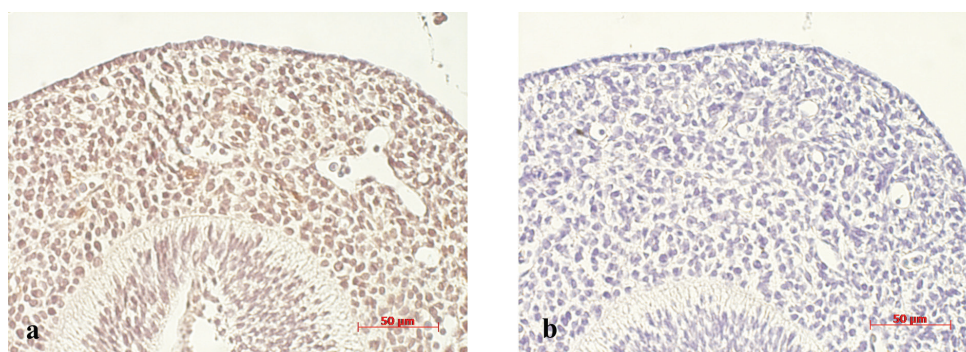


Figure 7. Transverse section of the isthmus region in human brain at CS 20; **a.** Strong expression of Pax2; **b.** Negative control, where during processing primary antibody was omitted.

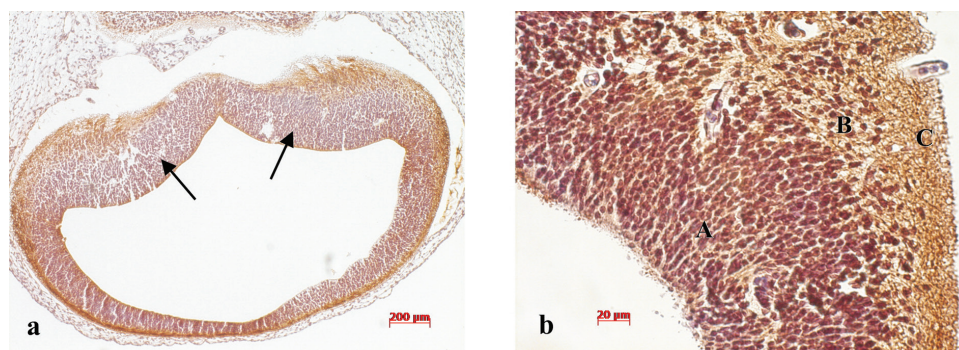


Figure 8. Transverse section of the brain of human embryos at CS 18; **a.** Expression of Pax2 in the wall of the diencephalon (arrows); **b.** Expression of Pax2 in the (A) ventricular, (B) mantle, and (C) marginal layers of the cerebellar plate.

Pax2 protein expression increased and staining was stronger during CS 16–20 compared to CS 10 (Table 1). Furthermore, some regional differences in Pax2 staining were seen in these later stages (CS 16–20) where the wall of the forming spinal cord has differentiated into the ventricular, mantle and marginal zones. Differences were noted in the staining intensity between the dorsal and ventral parts: in the dorsal part of the developing spinal cord, expression of

Pax2 was seen in all three layers at CS 16–20, but in the ventral part, staining was weak in the ventricular layer and Pax2 protein expression was mainly seen in the mantle and marginal layers. Spatial differences in the expression of Pax2 in the forming spinal cord of human embryos have also been found by other researchers. Terzic et al. [19] determined Pax2 expression in human embryos at CS 17 in the outer parts of the ventricular zone of the developing spinal cord,

while Pax2 signal was missing in the ventral quarter of the ventricular zone. More extensive reaction was also described in the dorsolateral plates than in the ventrolateral plates [19]. Tellier et al. [18] detected Pax2 expression in the dorsal part of the germinative zone in human embryos of CS 14–18, and some cells of the alar plate, but not in the forming anterior horn. Spatially restricted expression of Pax2 in the developing spinal cord has also been demonstrated in studies on mice [10] and chickens [2]. Thus, according to these findings, Pax2 can be considered one of the key regulators in the combinatorial action of transcription factors which determine the fate of specific populations of early neurons.

In our study, Pax2 protein expression was also found in all the 3 brain vesicles, prosencephalon (forebrain), mesencephalon (midbrain) and rhombencephalon (hindbrain). In early stages, CS 10–14, Pax2 staining in brain vesicles was relatively strong, especially when compared to that seen in the developing spinal cord in the same young embryos. The most significant difference was noted at the earliest stage — CS 10 (Table 1). However, at later stages, CS 16–20, the strongest Pax2 expression was found at the junction of the midbrain and the hindbrain (Fig. 6a). This is in concordance with studies on chicken and mice embryos, where early expression of Pax2 has been detected in the mesencephalon and the anterior hindbrain, but subsequently Pax2 expression was restricted to the mid-hindbrain region [1, 14, 23]. In human embryos from day 32 to 50 (CS 14–19), Pax2 signal has been detected in the hindbrain, but not in the midbrain or the forebrain [18]. In studies on human 8-week embryos (CS 22), Terzic et al. [19] have shown Pax2 expression to extend from the mesencephalic-rhombencephalic border along the entire rhombencephalon. In particular, strong expression was detected in the outer part of the ventricular zone at the junction of the caudal mesencephalon and rostral rhombencephalon [19]. This region is known as an isthmus organizer centre, which is believed to play an important role in the development of the mesencephalon and the cerebellum [8]. Several experimental animal studies have suggested that initiation and functioning of this organising centre crucially depends on Pax2 [1, 16, 22, 23], and that the regulatory role of Pax2 is an evolutionarily conserved feature for all vertebrates [14]. Furthermore, it has been shown that a mutation of the Pax2 gene in the isthmus leads to the loss of the midbrain and the cerebellum [3, 15, 22].

In our study, Pax2 was also determined in the developing cerebellum and diencephalon. Expression of Pax2 was seen in the ventricular, mantle and marginal layers in the developing cerebellar plate. In mouse embryos, Pax2 has specifically been found to be expressed in a subset of GABAergic cells, and it is proposed that these cells are migratory precursors of the molecular layer interneurons (basket and stellate cells) [7]. The diencephalon, which develops from the median part of the forebrain, is thought to consist of a roof plate and 2 alar (dorsal) plates but to lack floor and basal (ventral) plates [9, 11]. Expression of Pax2 in our study was found mostly in outer regions of the alar plates of the diencephalon. In mouse embryos, Pax2 expression has been determined in diencephalic cells at the border of region between diencephalon and telencephalon, more specifically in populations of cells within the developing septum, hypothalamus and eminentia thalami [4]. Furthermore, in mice, Pax2 has been shown to play an important role in the formation of the optic cup, which develops as an outgrowth of the diencephalon wall and therefore represents a direct extension of the brain tissue [4, 10, 12]. Expression of Pax2 in the developing optic cup has also been detected in human embryos [18, 19]. Thus, it can be suggested that, in human neurogenesis, Pax2 is associated with the establishment of the developing diencephalon as well with the migration and determining cells in the alar plates.

CONCLUSIONS

In conclusion, Pax2 protein expression in the developing spinal cord and brain of the human embryos mostly resembles descriptions of the role of Pax2 in the neurogenesis of animals where Pax2 is associated with the establishment of craniocaudal and ventro-dorsal boundaries within the developing neural tube and with migration of specific neural cell populations.

REFERENCES

1. Bouchard M, David G, Craven SE, Sun Q, Steinlein P, Busslinger M (2005) Identification of Pax2-regulated genes by expression profiling of the mid-hindbrain organizer region. *Development*, 132: 2633–2643.
2. Burrill JD, Moran L, Goulding MD, Saueressing H (1997) PAX2 is expressed in multiple spinal cord interneurons, including a population of EN1+ interneurons that require PAX6 for their development. *Development*, 124: 4493–4503.
3. Favor J, Sandulache R, Neuhäuser-Klaus A, Pretsch P, Chatterjee B, Senft E, Wurst W, Blanquet V, Grimes P, Spörle R, Schugart K (1996) The mouse Pax21^{Neu} muta-

- tion is identical to a human Pax2 mutation in a family with renal-coloboma syndrome and results in developmental defects of the brain, ear, eye, and kidney. *Genetics*, 93: 13870–13875.
4. Fotaki V, Price DJ, Mason J (2008) Newly identified patterns of Pax2 expression in the developing mouse forebrain. *Dev Biol*, 8: 79.
 5. Gerard M, Abibtol M, Delezoide AL, Dufier JL, Mallet J, Vekemans M (1995) Pax genes expression during human embryonic development, a preliminary report. *C R Acad Sci III*, 318: 57–66.
 6. Lee SB, Doberstein K, Baumgarten P, Wieland A, Ungerer C, Bürger C, Hardt K, Boehncke WH, Pfeilschifter J, Mihic-Probst D, Mittelbronn M, Gutwein P (2011) PAX2 regulates ADAM10 expression and mediates anchorage-independent cell growth of melanoma cells. *PLoS One*, 6: e22312.
 7. Maricich SM, Herrup K (1999) Pax-2 expression defines a subset of GABAergic interneurons and their precursors in the developing murine cerebellum. *Inc J Neurobiol*, 41: 281–294.
 8. Nakamura H, Watanabe Y (2005) Isthmus organizer and regionalization of the mesencephalon and metencephalon. *Int J Dev Biol*, 49: 231–235.
 9. Nieuwenhuys R, Voogd J, van Huijzen C (2008) The human central nervous system. Section I. 4rd Ed. Springer, New York, pp. 3–165.
 10. Nornes HO, Dressler GR, Knapik EW, Deutsch U, Gruss P (1990) Spatially and temporally restricted expression of Pax2 during murine neurogenesis. *Development*, 109: 797–809.
 11. O'Rahilly R, Müller F (1987) Developmental stages in human embryos. Carnegie Institution of Washington, Washington, pp. 1–306.
 12. Otteson DC, Shelden E, Jones JM, Kameoka J, Hitchcock JM (1998) Pax2 expression and retinal morphogenesis in the normal and Krd mouse. *Dev Biol*, 193: 209–224.
 13. Palmieri C, Gojis O, Rudraraju B, Stamp-Vincent C, Wilson D, Langdon S, Gourley C, Faratian D (2013) Expression of steroid receptor coactivator 3 in ovarian epithelial cancer is a poor prognostic factor and a marker for platinum resistance. *Br J Cancer*, 108: 2039–2044.
 14. Pfeffer PL, Payer B, Reim G, di Magliano MP, Busslinger M (2002) The activation and maintenance of Pax2 expression at the mid-hindbrain boundary is controlled by separate enhancers. *Development*, 129: 307–318.
 15. Schwarz M, Alvarez-Bolado G, Dressler G, Urbanek P, Busslinger M, Gruss P (1999) Pax2/5 and Pax6 subdivide the early neural tube into three domains. *Mech Dev*, 82: 29–39.
 16. Schwarz M, Alvarez-Bolado G, Urbanek P, Busslinger M, Gruss P (1997) Conserved biological function between Pax-2 and Pax-5 in midbrain and cerebellum development: evidence from targeted mutations. *Dev Biol*, 94: 14518–14523.
 17. Stoykova A, Fritsch R, Walther C, Gruss P (1996) Forebrain patterning defects in Small eye mutant mice. *Development*, 122: 3453–3465.
 18. Tellier AL, Amiel J, Delezoide AL, Audollent S, Auge J, Esnault D, Encha-Razavi F, Munnich A, Lyonnet S, Vekemans M, Attie-Bitach T (2000) Expression of the Pax2 gene in human embryos and exclusion in the Charge syndrome. *Am J Med Genet*, 93: 85–88.
 19. Terzic J, Muller C, Gajovic S, Saraga-Babic M (1998) Expression of PAX2 gene during human development. *Int J Dev Biol*, 42: 701–707.
 20. Terzic J, Saraga-Babic M (1999) Expression pattern of Pax3 and Pax6 genes during human embryogenesis. *Int J Dev Biol*, 43: 501–508.
 21. Thompson JA, Ziman M (2011) Pax genes during neural development and their potential role in neuroregeneration. *Prog Neurobiol*, 95: 334–351.
 22. Urbanek P, Fetka I, Meisler M, Busslinger M (1997) Cooperation of Pax2 and Pax5 in midbrain and cerebellum development. *Dev Biol*, 94: 5703–5708.
 23. Viera C, Garcia-Lopez R, Martinez S (2006) Positional regulation of Pax2 expression in mesencephalic and diencephalic alar plate. *Neuroscience*, 137: 7–11.
 24. Wehr R, Gruss P (1996) Pax and vertebrate development. *Int J Dev Biol*, 40: 369–377.