Università degli Studi di Cagliari

DOTTORATO DI RICERCA

Scienze e Tecnologie per l'Innovazione

Ciclo XXIX

TITOLO TESI

Immunohistochemical markers of stem/progenitor cells in the developing human cerebral cortex

Settore/i scientifico disciplinari di afferenza

MED/08

Presentata da	Laura Vinci
Coordinatore Dottorato	Prof. Roberto Orrù
Tutor	Prof. Rossano Ambu

Esame finale anno accademico 2015 – 2016
Tesi discussa nella sessione d'esame marzo – aprile 2017

Index

Abstract

The development of the human cerebral cortex represents a delicate moment of embryogenesis. The ventricular zone (VZ) and the subventricular zone (SVZ) are considered the “stem/progenitor cells niches” of the developing cerebral cortex. As the majority of studies of brain development have been focused mainly on animal models, this study was focused on normal human cerebral cortex development, in particular on the stem/progenitor cells markers that may play a key role during human corticogenesis. To this end, samples from cerebral cortex were obtained from 20 human fetuses from 9 up to 40 weeks of gestation. Each sample was formalin-fixed, paraffin embedded and immunostained with several markers including WT1, Sox2, Pax6, Vimentin, Nestin, Sox11, Pax2, NF, NSE, Synaptophysin, GFAP and S100B. Five important markers of radial glial progenitor cells were evidenced during the first half of gestation: Sox2, Pax6, Vimentin, Nestin and WT1. Vimentin, Nestin and WT1 were expressed in radial glia fibers which represent the most important guide for the radial migration of newborn neurons. Instead, Pax6 immunoreactivity was detected in the VZ and SVZ, being express in radial glia cell bodies. Sox2 was expressed by the stem/progenitor cells of the VZ and SVZ including radial glia and intermediate progenitor cells, and by migrating newborn neurons. Pax2 and Sox11 expression were detected in the progenitor cells of VZ and SVZ, and in the migrating newborn neurons. Immunoreactivity for S100B, GFAP, NF and Synaptophysin, was mild or totally absent in the first half of gestation. These data reflect the lack of maturation of glial and neuronal cells in the early phases of human corticogenesis. Future studies are needed to detect differences in the mRNA expression patterns of these markers in order to better evaluate their role in cell proliferation and differentiation during human intrauterine development.

1. Introduction

The complex organization of the human cerebral cortex has been proposed as the crowning achievement of human evolution distinguishing human beings from other animals species. Indeed, the cerebral cortex plays a key role in cognitive functions, intelligence language and consciousness, motor abilities, memory and sensory perceptions. For these reasons, neurodevelopment represents a very delicate moment of embryogenesis being involved on precisely orchestrated sequence of molecular and cellular events. Premature interruption of any of these events during human brain development may cause an imbalance between excitatory and inhibitory circuits and is responsible of significant motor, cognitive, behavioral or sensory deficits in childhood. Preterm infants may have a major risk to develop psychiatric illnesses and neurodevelopmental disorders including autism spectrum disorders, epilepsy, schizophrenia and neurodegenerative diseases later in life [1;2].

Over the past decade, the study of stem cells and neurogenesis has been the focus of many research groups, due to the possible role in the development and treatment of several neurologic disorders, including epilepsy, Parkinson's disease, and Alzheimer's disease [3;4;5].

A new branch of medicine called "regenerative medicine", aims to exploit the enormous potential of stem cells to produce new tissues to replace damaged ones in the adult organism [6]. However, stem cells research caused a great debate in society due to the numerous technical and ethical problems.

Seeing as the majority of studies of brain development has been focused mainly on animal models, this work is focused on normal human cerebral cortex development, in particular on the stem/progenitor cells markers that may play a key role during human corticogenesis. These molecular markers may represent possible targets of a potential regenerative "physiological" therapy in the first weeks of postnatal life.

1.1 Human corticogenesis

1.1.1 The embryonic and fetal cerebral cortex development

The human nervous system is subdivided into central nervous system (CNS) and peripheral nervous system (PNS). CNS represents the largest part of the nervous system, including the brain and the spinal cord. The CNS appears at the beginning of the third week of gestation as a slipper-shaped plate of thickened ectoderm. It arises from the neural plate, a specialized region of the ectoderm [7]. During embryonic development, the neural plate folds forming the neural tube (Fig. 1). Fusion begins in cervical region and then proceeds in cephalic and caudal direction. Once fusion is started, the open ends of the neural tube form the cranial and caudal neuropores which communicate with the overlying amniotic cavity [7]. The closure of neuropores occurs approximately at about the 24th and the 27th day of gestation, respectively (Fig. 1). The internal cavity of the neural tube will give rise to the ventricular system and the whole neural tube will differentiate into two major subdivisions: brain and spinal cord.

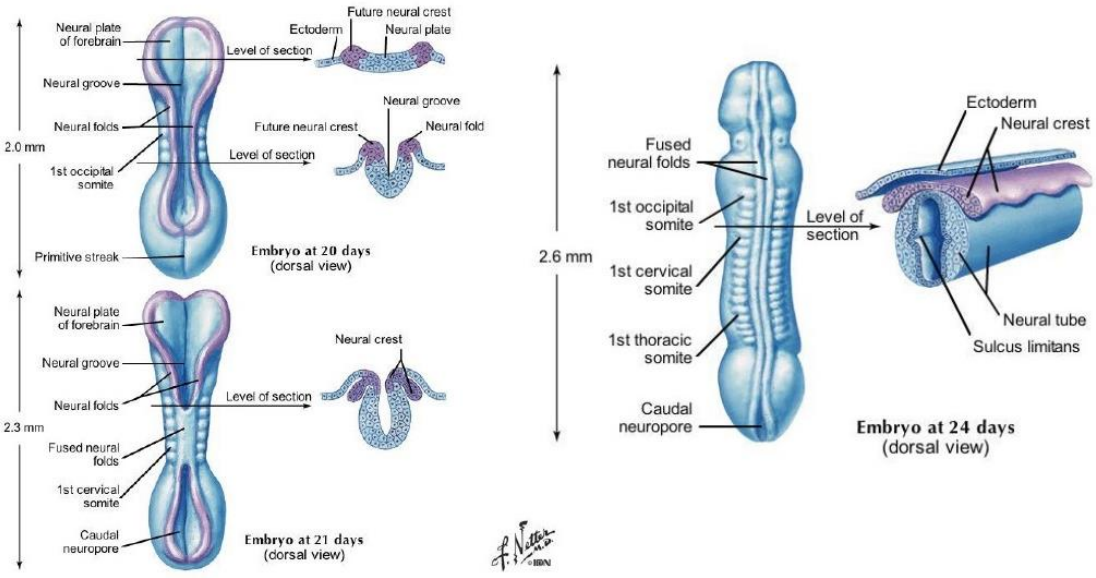

Figure 1. Human neural tube development at 21 and 24 days of gestation. [© Netter Atlas of Human Embriology]

The three major divisions of the brain appear at the end of the 4th week of gestation: the prosencephalon (forebrain), the mesencephalon (midbrain) and the rhombencephalon (hindbrain). During the 5th week of gestation, the prosencephalon will be subdivided into telencephalon and diencephalon (Fig. 2). The telencephalon gives rise to the primitive cerebral hemispheres dorsally and the basal ganglia ventrally with the cavity becoming the lateral ventricles. The rhombencephalon consists of two parts: the metencephalon, which later forms the pons and cerebellum, and the myelencephalon (Fig. 2).

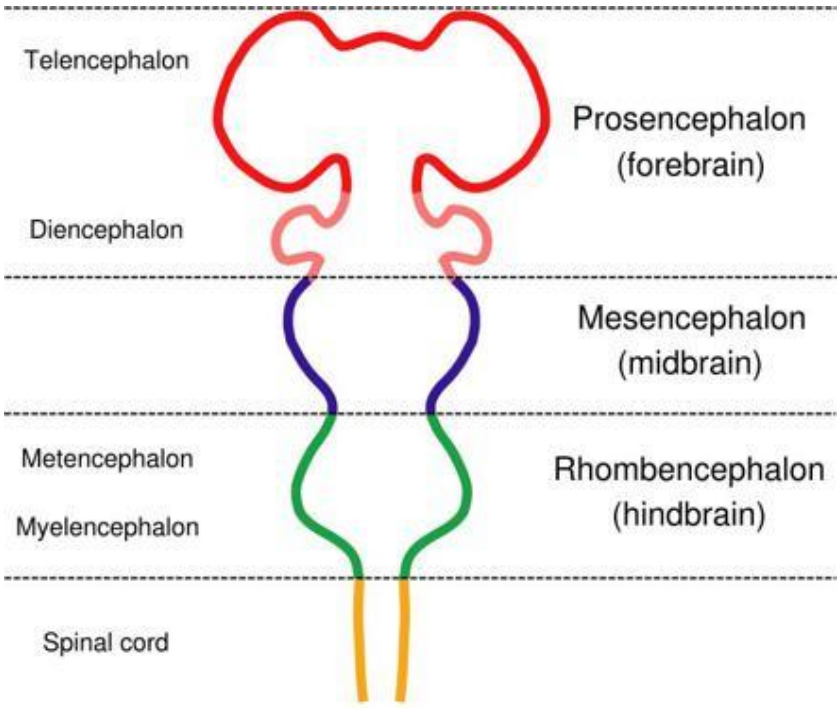

Figure 2. Human brain at 5th week of gestation

The neuroectodermal cells dorsally located along each side of the neural plate and not involved in the development of the neural tube form the neural crest. The neural crest cells migrate extensively to generate differentiated cell types that include the neurons and glial cells of the sensory, sympathetic, and parasympathetic nervous systems, the epinephrine-producing (medulla) cells of the adrenal gland, the pigment-containing cells of the epidermis, and many of the skeletal and connective tissue components of the head [7].

Human cerebral cortex development is a process that begins in the 3rd gestational week with the differentiation of the neural progenitor cells and extends at least through late adolescence, arguably throughout the lifespan [8].

From a biological perspective, it is possible to identify six distinct phases of embryonic and fetal cerebral cortex development:

1. Mitotic cell division
2. Cell proliferation
3. Cell migration
4. Cell differentiation
5. Neuronal connectivity
6. Synaptogenesis
7. Synaptic pruning/programmed cell death (PCD)

The first 3 phases span throughout the first half of gestation and are characterized by the genesis of new neurons by *mitotic cell division* (neurons multiply at a rate of 250,000 cells per minute) and their *proliferation* and *migration* toward the brain surface [9]. Cortical neurons arise from neuroepithelial cells of the ventricular zone and fastly proliferate since the 10th gestational week until the 22nd week, then follow a phase of slower proliferation. This period is associated with a noticeable cortical growth both in thickness and surface area [10].

The formation of *neuronal connectivity* spans from mid-gestation throughout 2 years postnatally. This phase is the most significant time of cytological *differentiation* of the cerebral cortex being involved in the growth of neuronal dendrites and axons, the generation and expansion of astrocytes, oligodendrocytes and microglial cells, the formation of synapses, and the development of the vascular system. The new connections induce an excessive tangential expansion of the cortex [11], causing an increase in cortical stress and consequent folding of the cerebral cortex [12].

The last two phases, *synaptogenesis* and *synaptic pruning*, span throughout the entire lifetime and they are associated with the formation of a few new connections and mainly with the removal of unnecessary neuronal structures [13]. Neuronal survival is regulated via selection processes that require synaptic activation of newborn cells. The human cortex remains plastic throughout these phases.

1.1.2 The mature cerebral cortex

The average adult human brain has a volume of around 1260 cm^3 in men and 1130 cm^3 in women, a total surface area of 1820 cm^2, and an average cortical thickness of 2 to 4 millimetres [14]. It contains approximately 100 billion neurons, 16 billion of which are located in the cerebral cortex (including subcortical white matter). Each cortical neuron has an average 7000 synaptic connections to other neurons, resulting in a total of 0.15 quadrillion synapses and more than 150,000 km of myelinated nerve fibers [15]. The brain includes the brainstem, the midbrain, the cerebellum and the cerebral hemispheres (right and left). The superficial part of the cerebral hemispheres is known as the cerebral cortex; in addition, the cerebral hemispheres include the white matter and three deep nuclear formations.

The cerebral cortex is subdivided into four regions, which cover both hemispheres: the *frontal lobe*, containing dopamine-sensitive neurons associated with reward, attention, short-term memory tasks, planning, and motivation; the *parietal lobe*, involved in integrating sensory information from the various senses; the *temporal lobe*, involved in visual memory and language comprehension; and the *occipital lobe,* involved with the sense of sight (Fig. 3).

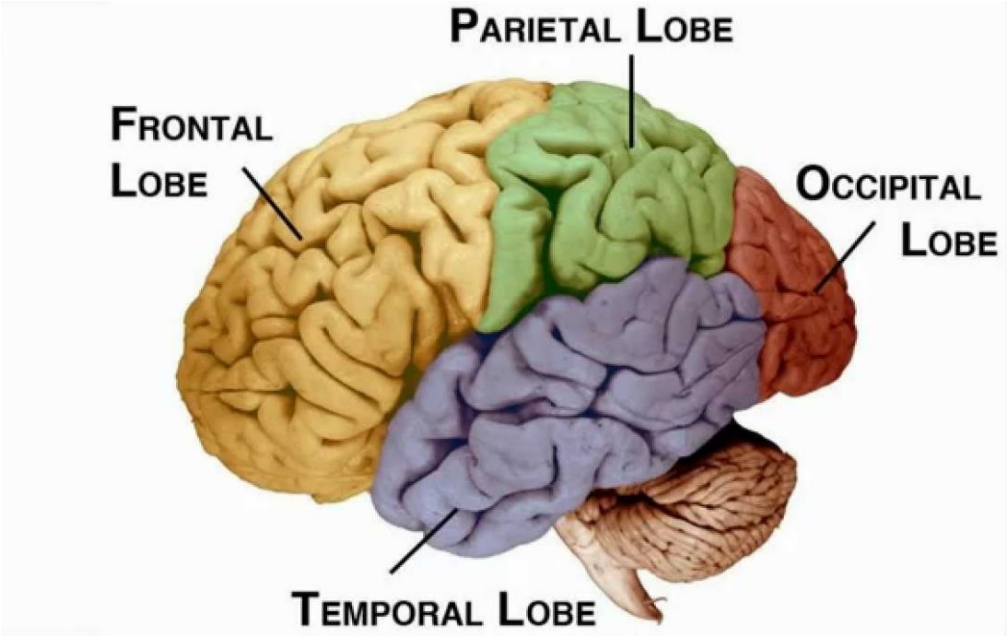

Figure 3. Lobes subdivision of mature human cerebral cortex

The human cerebral cortex consists of a thin mantle of gray matter enclosing the underlying white matter and is characterized by two major differentiated cell types: *neurons* and *glial cells*. Neurons transmit information through action potentials and neurotransmitters to other neurons, muscle cells or gland cells. Glial cells play important roles in CNS providing a critical support and protection roles maintaining homeostasis for a normal neuronal functioning and survival. In the CNS, glial cells include oligodendrocytes, astrocytes, ependymal cells and microglia. In contrast to the neuroectodermal origin of the majority of CNS cells, microglia - the principal active immune defense cells of the brain - derives from the mesoderm [16]. In recent years, microglia has been clarified to originate from two sources: the yolk sac and myeloid precursors [17;18].

The gray matter consists of neuronal cell bodies, dendrites and axons, glial cells and capillaries, while the white matter is composed of bundles of myelinated nerve cell projections, which connect various gray matter areas of the brain to each other, and carry nerve impulses between neurons.

The neurons of the cerebral cortex are grouped into six main layers that, from the pial surface to white matter, are:

1. *Layer I* - the molecular layer, that contains few scattered neurons and consists mainly of extensions of apical dendrites of pyramidal neurons, horizontally oriented axons and glial cells;
2. *Layer II* - the external granular layer, that consists in small pyramidal neurons and several stellate neurons;
3. *Layer III* - the external pyramidal layer, that contains pyramidal neurons, as well as non-pyramidal neurons with vertically oriented intracortical axons that increase in size from the outer to the inner boundary of this layer. Layer III is the main source of cortico-cortical efferents. Moreover, layers I through III are the main target of interhemispheric cortico-cortical afferents;
4. *Layer IV* - the internal granular layer, that contains mainly stellate neurons and a few interneurons and pyramidal neurons;
5. *Layer V* - the internal pyramidal layer, that contains mainly large pyramidal neurons interspersed with interneurons. Pyramidal neurons fibers project to subcortical structures such as the basal ganglia, brainstem and spinal cord;
6. *Layer VI* - the polymorphic or multiform layer, that contains mainly multiform neurons and few large pyramidal neurons; layer VI sends efferent fibers to the thalamus, establishing a very precise reciprocal interconnection between the cortex and the thalamus.

The cerebral cortex is divided into different cytoarchitectonic areas (52 Brodmann areas) according to the different thickness of each layer, neuronal morphology in the layers and distribution of axonal bundles (Fig. 4). For istance, in the Brodmann area 4 and Brodmann area 6, refering to the primary motor cortex and premotor cortex respectively, the number of pyramidal cells is greater than interneurons. Layers from the 2^{nd} to the 6^{th} appear

almost completely formed by pyramidal cells of different size, with those of greater size disposed in the deeper layers (Fig. 4).

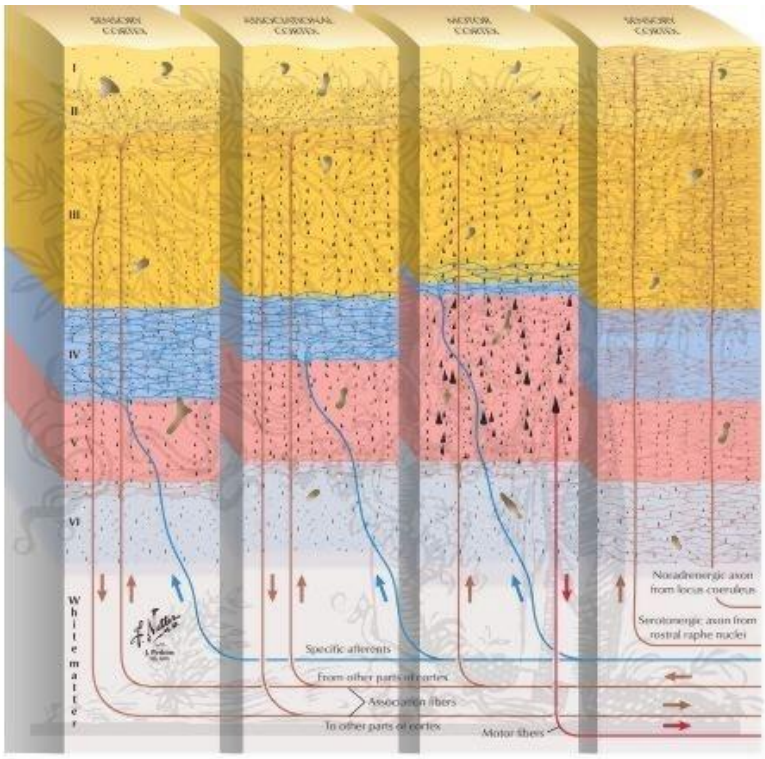

Figure 4. The six cortical layers of mature cerebral cortex differ according to the cytoarchitectonic areas. [© Netter Atlas of Human Anatomy]

1.2 Stem/progenitor cells

1.2.1 Unique Properties of Stem Cells

At the end of the nineteenth century, a theoretical postulate describe for the first time the self-regenerative capacity of some tissues. Tissues that consist for the most part of cells with a limited duration of life, require a quantity of cells responsible to support the renewal of the functional cell types for the entire life of the organism. Therefore, several studies were developed to evaluate the major characteristics of stem cells and the different functions and characteristic of embryonic and adult stem cells.

Stem cells have three general properties [19]:

- Self-renewal: they are capable of dividing and self-maintenance for long periods through the process of cell division;
- Unspecialized: one of the fundamental properties of a stem cell is that is not a specialized cell; it has not any tissue-specific structures that allow it to perform specialized functions;
- Potency: in physiological or experimental conditions, stem cells can give rise to specialized cells in a process called differentiation.

Contrary to most cells of the body, which are committed to perform a specific function, a stem cell has a unique capacity to renew itself and to give rise to many specialized cell types. Moreover, a stem cell is uncommitted and remains uncommitted, until it receives a signal to develop into a specialized cell.

Stem cells can undergo to two different mitotic cell division: *symmetrical and asymmetrical division* [20] (Fig. 5).

During the *symmetric cell division*, can be generate two daughter cells identical to mother cell and capable of self-renewal, or two daughter cells with the capacity of differentiation. The first case occurs whenever is required an increase in the number of stem

cells such as during embryogenesis or in tissue repair. Cancer stem cells use this type of mitotic division (Fig. 5).

During the *asymmetric cell division*, mother cell give rise to two daughter cells with different cellular fates: one daughter cell remains stem cell identical to mother cell, the other one becomes a differentiated progeny cell or stem cell with a limited capacity of differentiation (Fig. 5).

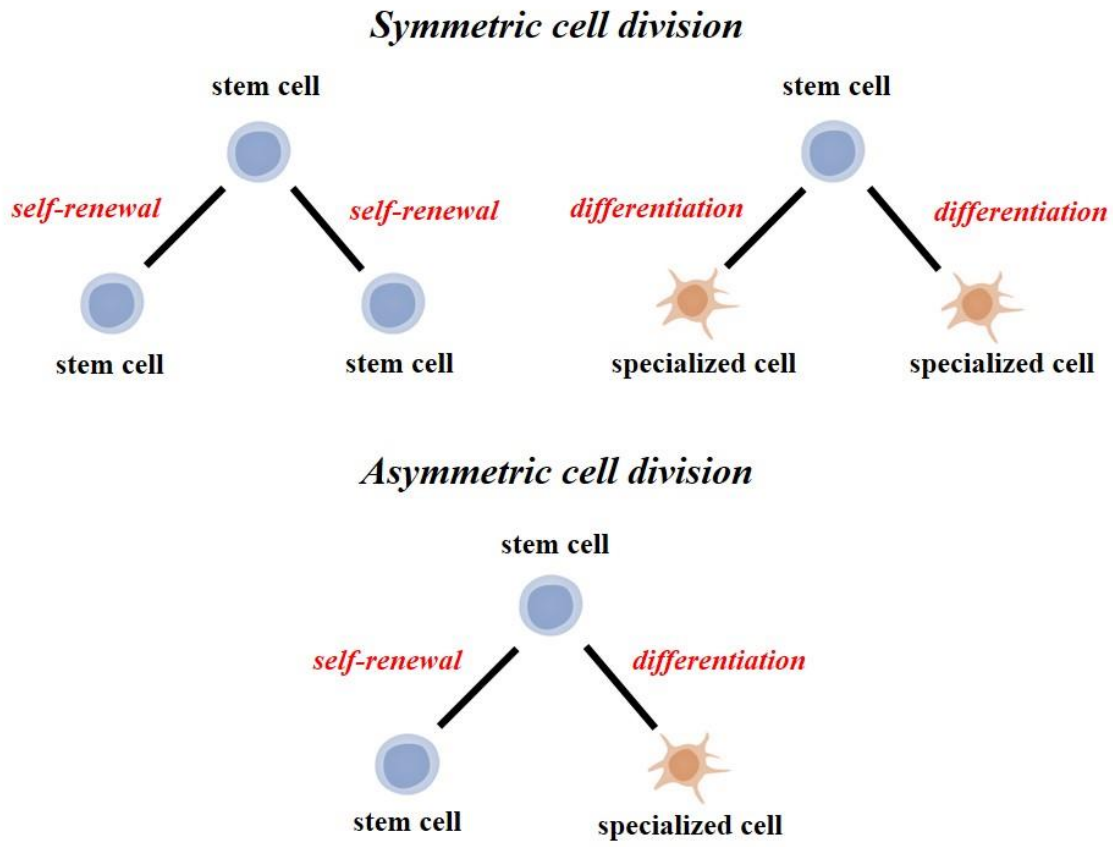

Figure 5. Symetric and asymentric cell division

Stem cells have different potentials to differentiate into different cell types [21]. According to their differentiation potential, stem cells are classically divided into:

- *Totipotent stem cells:* stem cells able to differentiate into any embryonic and extraembryonic cell types including primitive germ-line stem cells. These cells are present in embryos at the 4-8 cell stage, 1-3 days after fertilization;
- *Pluripotent stem cells:* they are characterized by a differentiation potential to all cell types of the adult organism and to self-renewal. These cells appear at the blastocyst stage (4-14 days after fertilization) and they are capable of differentiating into embryonic tissues organized in three different germ layers (ectoderm, mesoderm and endoderm);
- *Multipotent stem cells:* they have the ability to replicate and remain in the culture but losing the ability to self-renewal. These cells differentiate into different tissues belonging only to the same germ layer. Adult stem cells belong to this category. For example, bone marrow stem cells are able to give rise to blood cells, but are not able to give rise to any other cell types [21];
- *Unipotent stem cells:* these cells are present in adult tissues. They maintain self–renewal property and they can differentiate only in one cell type of the tissue to which they belong, ensuring repair and maintenance.
-

1.2.2 Stem/progenitor cells niche of the developing cerebral cortex: the ventricular and the subventricular zones

Cortical neurogenesis involve cell proliferation, migration and differentiation and leads to the final creation of neural and glial cells to form the characteristic six-layered cortical structure from inside to outside [8]. During neurogenesis neuronal and glia cells are

generated from a common source, the proliferating neuroepithelial cells (NECs), which arise from the neural tube. The differentiation of the neural progenitor cells is orchestrated by internal signals that are controlled by genes that carry information for all the structures and functions of a cell, and by external epigenetic signals including hormones and molecules secreted by other cells. Extrinsic factors, believed to be essential for maintenance and proliferation of the neural stem/progenitor cells pool, include Fibroblast Growth Factor (FGF) [22], Epidermal Growth Factor (EGF) [23], Sonic Hedgehog (SH) [24], and Wnt family [25].

During the early stage of the neural tube development, NECs form the columnar monolayered epithelium that, at the end of neurulation, gives origin to the pseudostratified epithelium [26]. The characteristic pseudostratification is mainly due to interkinetic nuclear migration. NECs undergo symmetric proliferative division leading to thickening of the neuroepithelium and growth of the neocortex [27].

Cortical neurogenesis begins around gestational week 5 in the *ventricular zone* when NECs undergo asymmetric division: by self-renewing itself, one mother cell (NEC or apical radial glia) gives rise to one identical cell capable of self-renewal; the other daughter cell becomes either an apical intermediate progenitor, a basal progenitor cell or a newborn neuron. In the further stages of neurogenesis, NECs are progressively replaced by apical radial glia (aRG) cells that begin to express astroglial markers and form radial fibers extending from their apical and basal poles [28]. At gestational week 6 the humans cerebral cortex is formed from two layers: a thick proliferative ventricular zone and a narrow, cell-sparse pial zone. The latter contains migrating newborn neurons [29] and it will eventually become cortical layer 1 [30].

The underlying subplate zone contains both, interneurons and postmigratory neurons, which transiently connect with incoming axons until the cortex is ready to receive them [31].

Like NECs, aRG cells undergo interkinetic nuclear migration in the ventricular zone. During neurogenesis progression, aRG cells switch from proliferation to differentiation (Fig. 6).

Apical intermediate progenitors, basal intermediate progenitors and basal radial glia can be generated either from NECs or aRG, and from themselves. Both types of basal progenitor cells are not attached to the ventricular surface and do not undergo interkinetic nuclear migration.

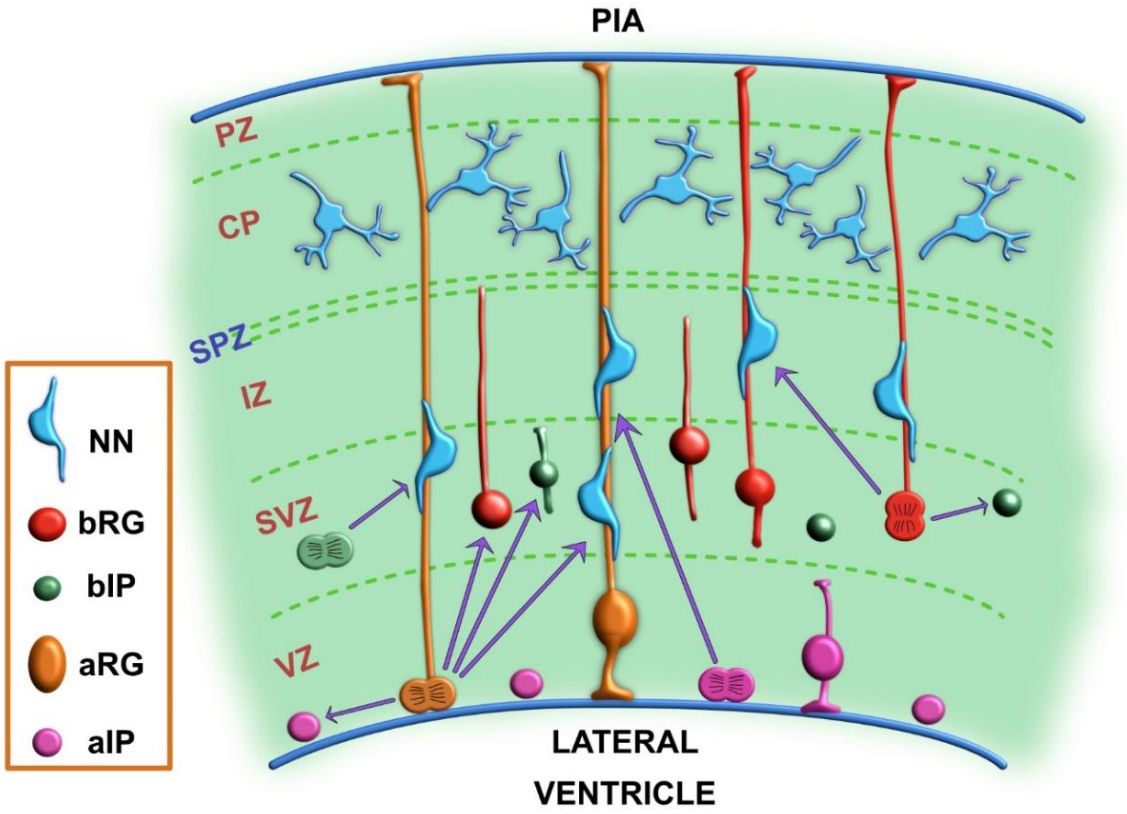

Figure 6: Schematic representation of stem/progenitor cells in human cerebral cortex niche in the early stage of gestation: ventricular zone (VZ), subventricular zone (SVZ), intermediate zone (IZ), subplate zone (SPZ), cortical plate (CP), pial zone (PZ); apical radial glia (aRG), apical intermediate progenitor (aIP), basal radial glia (bRG), basal intermediate progenitor (bIP), newborn neuron (NN).

Around gestational week 7, the accumulation of basal progenitor cells creates the *subventricular zone*, a distinct new germinal layer located above the ventricular zone [32;33]. Apical intermediate progenitors maintain contact only with the ventricular surface.

Apical progenitor cells in the ventricular zone and basal progenitor cells in the subventricular zone are generally considered to represent the major source of cortical neurons [34;27] (Fig. 6). For that reason, these two zones represent stem/progenitor cells niche of the developing cerebral cortex [35].

Radial glia cells play a key role during CNS development thank to their ability to generate neurons, astrocytes, oligodendrocytes and ependymal cells. Moreover, their fibers serve as a scaffold for neuronal migration from the ventricular zone toward definitive destination in the cortical plate. Around gestational week 10 cortical neurons increase proliferation in the ventricular and subventricular zones and then they decrease at around gestational week 22. At 10 weeks of gestation, the developing human cerebral cortex consists of seven different zones: the ventricular zone, the subventricular zone, the intermediate zone, the subplate zone, the cortical plate and the pial zone (Fig. 7).

Whereas the pial zone will eventually form the cortical layer 1, the cortical plate will give rise the others 5 layers. The earliest newborn neurons are destined to become the innermost layer 6, the last newborn neurons will become the outer layer 2. Therefore, neurons of the inner cortical layers originate from apical radial glial cells in the ventricular zone, whereas later neurons in the superficial layers progressively originate from basal progenitor cells in the subventricular zone [36]. The subplate zone disappears by the first postnatal month whereas subplate neurons persist in adult brain white matter as interstitial neurons. The intermediate zone consists of radially migrating cells and long- range axons [37]. At the end of gestation, this zone will become white matter tissue. Once that neurogenesis is complete, the radial glia differentiate into glial lineage.

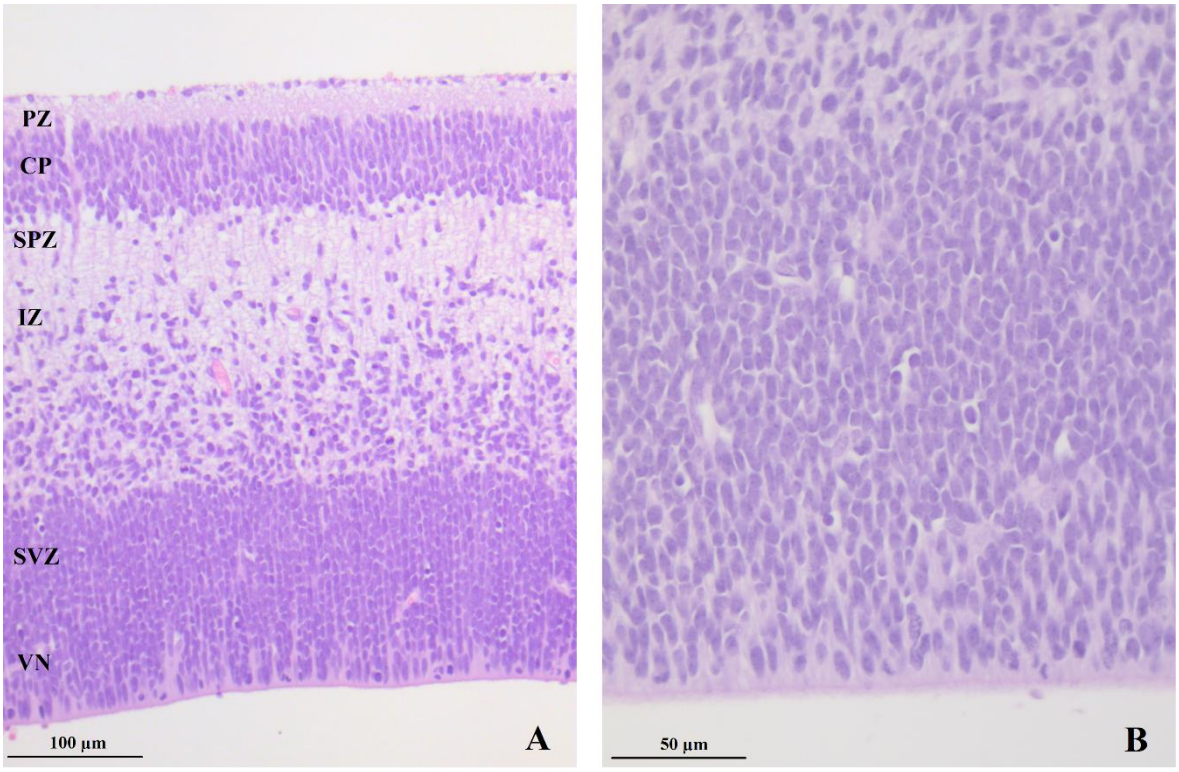

Figure 7: A) Different regions of human cerebral cortex at gestational week 10: the ventricular zone (VZ), the subventricular zone (SVZ), the intermediate zone (IZ), the subplate zone (SPZ), the cortical plate (CP) and the pial zone (PZ); B) higher magnification of stem/progenitor cells niche of VZ and SVZ.

1.2.3 Stem/Progenitor cells niches in adult human brain

For almost a century, the scientific community considered the idea that neurogenesis exhausted in the early stages of post-embryonic development as an axiom of neurobiology. Neurologists were practicing their profession under the doctrine established in the early 20th century by histologist Ramon y Cajal:

"Once the development was ended, the founts of growth and regeneration of the axons and dendrites dried up irrevocably. In the adult centers, the nerve paths are something fixed, ended, and immutable. Everything may die, nothing may be regenerated. It is for the science of the future to change, if possible, this harsh decree".

(Ramon y Cajal, 1913).

In the last twenty years, the proof of the existence of neurogenic niches in adult human brain has finally demolished this dogma.

The first reports of cell division and differentiation in the adult brain emerged from experimental studies on mouse brain development by Leblond and colleagues in the early 1960s [38]. In the mid-1960s, Altman et al [39;40] first showed the rostral migratory stream of cells between the SVZ and olfactory bulbs [41]. They also observed dividing cells in the subgranular zone and neurogenesis in the dentate gyrus of rat adult brain [40].

In the last few decades, neurogenesis in adult human brain has been demonstrated through several techniques including stereological techniques for labeling dividing cells (BrdU) and neurons (NeuN), and carbon^{14} dating techniques. In the late 1990s, Erickson demonstrated that new neurons are generated from dividing progenitor cells in the dentate gyrus of human adult [42].

In summary, adult stem/progenitor cells are responsible for the generation of new neurons in two main niches of the adult human brain: *the subventricular zone of the lateral ventricles* and *the subgranular zone of the dentate gyrus of the hippocampus* [43]. In the dentate gyrus, newborn cells differentiate into excitatory granule cells. Stem/progenitor cells in the subventricular zone generate restricted neural progenitor cells that migrate through a glial cell scaffold via the rostral migratory stream towards the olfactory bulb and then differentiate into distinct types of olfactory neurons [44].

The functional significance of these new neurons is uncertain. Several studies showed that newborn neurons contribute to olfactory- and hippocampus-dependent learning and memory [45]. Moreover, hippocampal adult neurogenesis is important for related mechanisms of neural plasticity such as long-term potentiation (LTP) [46]. In recent years, adult neurogenesis has been suggested to play a critical role in brain homeostasis and disease. Deficient neurogenesis has been implicated in the pathogenesis of multiple neurological and psychiatric diseases [47] including depression, epilepsy [48] and Alzheimer's disease [49].

Indeed, in recent years epigenetic factors have been proposed as a possible cause of neurogenesis disarrangement during gestation and of susceptibility to develop neurodegenerative disorders later in life, including Alzheimer's disease and Parkinson's disease [5].

1.3 Cortical stem/progenitor cells markers

Several immunohistochemical markers have been utilized in previous experimental studies for the identification of neural stem/progenitor cells in the developing cerebral cortex. This study was focalized on the immunohistochemical expression of some proteins that play key role in mammalian central nervous system development: Sox2, Sox11, WT1, Nestin, Vimentin, Pax2 and Pax6.

1.3.1 Sox2

Sox2 is a member of the Sry-related high mobility group (HMG) box (SOX) family of transcription factors. Proteins of the Sox family all share a highly conserved high-mobility-group (HMG) DNA binding domain [50]. The Sox gene family was first defined by the discovery of the mammalian testis-determining factor - Sry - involved in the regulation of embryonic development and in the determination of cell fate [51;52]. SOX2 play an important role during mammalian CNS development [53] and its expression was also reported in neural stem/progenitor cells located in adult neurogenesis niches: the subventricular zones of lateral ventricles and the subgranular zone of the hippocampus [54]. Recently, it has been suggested that Sox2 plays a critical role in initiating the neural induction and in maintenance of neural progenitor stem cell properties throughout neural differentiation [55]. Therefore, Sox2 is highly expressed in proliferating neural progenitor cells, maintaining the self-renewal property [55;56], and it is down-regulated upon differentiation to post-mitotic neuronal and glial cells [57]. Moreover, Sox2 act with the

transcription factors Oct4 and Nanog regulating the differentiation of stem/progenitor cells to specific lineages [58].

1.3.2 Sox11

Sox11 belongs to SoxC subfamily, is highly expressed in some subtypes of precursors and post-mitotic neurons being involved both embryonic [59] and adult neurogenesis [60;61]. This transcription factor regulates survival and axonal growth of embryonic mouse sensory neurons [62]. Recently, a key role of Sox11 in promoting neuronal differentiation has been described in the chick spinal cord, in the mouse hippocampus [61] and in the mouse cerebral cortex [63;64;65]. Moreover, Sox11 plays a crucial role in mouse corticogenesis through balancing dendritic morphogenesis with neuronal migration [66].

1.3.3 WT1

Wilms' Tumor 1 (WT1) protein is a zinc finger transcription factor encoded by the human gene WT1 involved in the onset of Wilms' tumor, the most common primary renal tumor in childhood [67]. WT1 gene has a length of ~ 50 kb and consists of 10 exons [68] and it shares a high degree of structural homology with the early growth response (EGR) transcription factor family [69;70]. The combination of alternative splicing of mRNA and RNA editing can generate more than 20 different gene products [71;72]. The corresponding isoform proteins differ in the selective domain for DNA binding: isoforms -KTS are potent transcriptional activators and preferentially bind to DNA while the isoforms +KTS have a role in RNA binding [73]. The WT1 protein regulates the transcription of several genes and acts both as activator and as transcriptional co-activator or as repressor of gene expression [74].

Studies on knock-out mice showed that WT1 is required for heart, spleen and adrenal glands development and for the development of the CNS [75-78]. WT1 is involved in the development of several human organs during embryogenesis [79;80], including developing

kidney [81;82]. Recent studies of our group have showed WT1 as an important marker involved in human CNS development [79] being expressed in radial glia cells in early phases of gestation [83].

1.3.4 Nestin

Nestin, a cytoskeleton-associated class VI intermediate filament (IF) protein, is a key regulator of various extracellular proteins that play important roles in cell growth and differentiation [84]. It has been reported *Nestin* involvement in adult and embryonic neurogenesis [85;86]. Nestin regulates neural stem cell migration via controlling the cell contractility in murine neurogenesis studies [87]. During embryogenesis, nestin expression is downregulated and gradually replaced by cell type-specific intermediate filaments such as Neurofilaments (NF) in neurons and Glial fibrillary acidic protein (GFAP) in glial cells [88].

1.3.5 Vimentin

Vimentin is a type III intermediate filament protein and acts as a crucial cytoskeletal component of mesenchymal cells being involved in cell migration and in epithelial-to-mesenchymal transition (EMT) [89;90]. During the reverse process, the mesenchymal to epithelial transition (MET), Vimentin is downregulated and, consequently, cell motility decreases and cells adopt epithelial characteristics.

Vimentin is overexpressed in various epithelial cancers, including prostate cancer, gastrointestinal tumors, CNS tumors, breast cancer, malignant melanoma, and lung cancer [91;92]. Moreover, Vimentin plays a key role during embryogenesis including CNS development [93]. The expression of this protein was detected in radial glia cells of neocortex and spinal cord in early stages of mammalian embryogenesis [94-96].

1.3.6 Pax2

PAX2 belongs to the highly conserved DNA-binding paired box domain (Pax) family which constitutes a group of developmental genes encode nuclear transcription factors which play an important role in early mammalian embryogenesis [97] including kidney development [98]. PAX2 is a target of transcriptional suppression by WT1 during normal kidney development [99] and it has been reported to be expressed during the formation of the CNS in experimental animal studies [100;101] including eye development [102]. Moreover, Pax2 support a role in neural patterning in human embryos being expressed in spinal cord e neocortex in early phases of gestation [103;104].

1.3.7 Pax6

Pax6 is a member of a family of transcription factors characterized by 384 base pair DNA sequence that encodes the paired domain (PD), and also contains a paired-type homeodomain (HD) and a transactivation domain (TA) [105;106]. PD and HD recognize distinct DNA targets sites and regulate different molecular mechanisms. Pax6 was initially found to be a master regulator of eye development [107;108]. Pax6 also plays a critical role in brain development [109;110]. The expression of Pax6 was found in radial glia cells of developing mammalian cerebral cortex [111;35] acting in cell proliferation and differentiation [112]. As it has already been described, radial glia cells give rise to intermediate progenitor cells in the ventricular and subventricular zones. Studies on animal models showed the transition from radial glia to intermediate progenitor cell is associated with upregulation of Tbr2 and downregulation of Pax6. Tbr2, a T-domain transcription factor, directs conversion of radial glia into basal precursors and guides neuronal amplification in the developing neocortex [113]. When the intermediate progenitor cells convert in postmitotic neurons, downregulation of Tbr2 and upregulation of Tbr1 have been observed, delineating differentiation pathway from radial glia [114].

2. Aim of the study

The aim of this study was to identify the stem/progenitor cell immunhistochemical markers located in the cortical neurogenesis niches during different human gestational ages. Because of this, the following stem/progenitor markers have been tested: WT1, Sox2, Pax6, Vimentin, Nestin, Sox11 and Pax2. Moreover, the expression of these markers has been compared with the expression of mature neurons and glia markers including Neurofilament (NF), Neuron Specific Enolase (NSE), Synaptophysin (Syn), Glial fibrillary acid protein (GFAP) and S100B.

Evaluating both stem/progenitor and mature cells markers allows the identification of the multiple stages of differentiation of neuronal and glial progenitors during gestation and it is also of great help in the better understanding the normal development of human cerebral cortex.

3. Materials and methods

The expression of different markers was investigated in frontal cerebral cortex from 20 human fetuses aging 9 up to 40 weeks of gestation that we received from the Obstetric Division of the University of Cagliari, as voluntary termination of pregnancy (VTOP) and as therapeutic and spontaneous abortion. Al the fetuses had no congenital malformations.

The frontal cerebral cortex of these fetuses has been sampled and histologically and immunohistochemically studied. Samples were fixed in 10% buffered formalin, routinely processed, and paraffin-embedded. Serial 3 µm-thick sections were obtained from each paraffin block; after dewaxing and rehydratation, one of these sections was stained with hematossilin-eosin, while the others were pre-treated for immunohistochemical analysis, with 10 minutes heat-induced epitope retrieval (*EnVision^{TM} FLEX Target Retrieval Solution Dako Denmark A/S, Glostrup, Denmark - High pH Code: K8004; Low pH Code: K8005*). Slides were then incubated for 20 minutes at room temperature with the antibodies reported in Table 1. Staining procedures were performed by Envision^{TM} FLEX+ (*Dako, Code: K8002*) Detection System and *AutostainerLink 48* instrument following dealer's instructions.

Antibody	Dilution	Source	Company	Code
WT1	1:100	mouse monoclonal 6F-H2	Dako	M3561
Pax2	1:400	mouse monoclonal 3C7	Abnova	H00005076-M01
S100B	1:2000	rabbit polyclonal	Dako	Z0311
Nestin	1:200	mouse monoclonal 10C2	Santa Cruz	SC-23927
Vimentin	1:500	mouse monoclonal 3B4	Dako	M7020
Sox2	1:50	mouse monoclonal E-4	Santa Cruz	SC-365823
NSE	1:200	mouse monoclonal BBS/NC/VI-H14	Dako	M0873
Synaptophysin	1:20	mouse monoclonal SY38	Dako	M0076
GFAP	1:100	rabbit polyclonal	Novocastra	NCL-GFAP-GA5
Neurofilament	1:50	mouse monoclonal 2F11	Dako	M0762
Pax6	1:50	mouse monoclonal	Santa Cruz	SC-53108
Sox11	1:100	mouse monoclonal CL0142	Abcam	ab154138

Table 1: Antibodies utilized in this study.

4. Results

4.1 Stem/progenitor cells markers

4.1.1 Sox2

From the 9th to the 12th gestational week, immunostaining for Sox2 was particularly strong in both ventricular and subventricular zones, associated with a weaker immunostaining in the intermediate zone and in the cortical plate cells. At these time point, especially at the 9th week of gestation, the ventricular and subventricular zones have larger thickness in comparison with the other zones of developing cerebral cortex (Fig. 9). Sox2 nuclear immunoreactivity was detected both in radial glia and intermediate progenitor cells in the stem/progenitor cells niches and also in the migrating newborn neurons of both the intermediate zone and the cortical plate (Fig. 9 and Fig. 10).

From the 15th to the 19th gestational week, the ventricular and subventricular zones become progressively thinner and the cortical plate begins to increase its thickness. At these gestational ages, Sox2 nuclear immunostaining is strong in the niches and increase in the intermediate zones as compared to the previous weeks, evidencing an increase of migrating newborn neurons toward the pial zone (Fig. 10A). Scattered Sox2-reactive cells were found in the cortical plate (Fig. 10B).

At the 21st weeks of gestation Sox2 immunoreactivity showed a lesser intensity and a reduced distribution in all the cerebral cortex zones (Fig. 11).

Between the 24th and the 30th gestational week, the ventricular and subventricular zones are thinner than in the previous weeks. Immunostaining for Sox2 was detected mainly in these two niches. Scattered Sox2-reactive cells were detected in intermediate zone and in cortical plate (Fig. 12A).

From the 34th to the 40th week of gestation, Sox2 immunoreacticity was detected in scattered cells in the ventricular zone. No reactivity for Sox2 was found in the cortex layers (Fig. 12B).

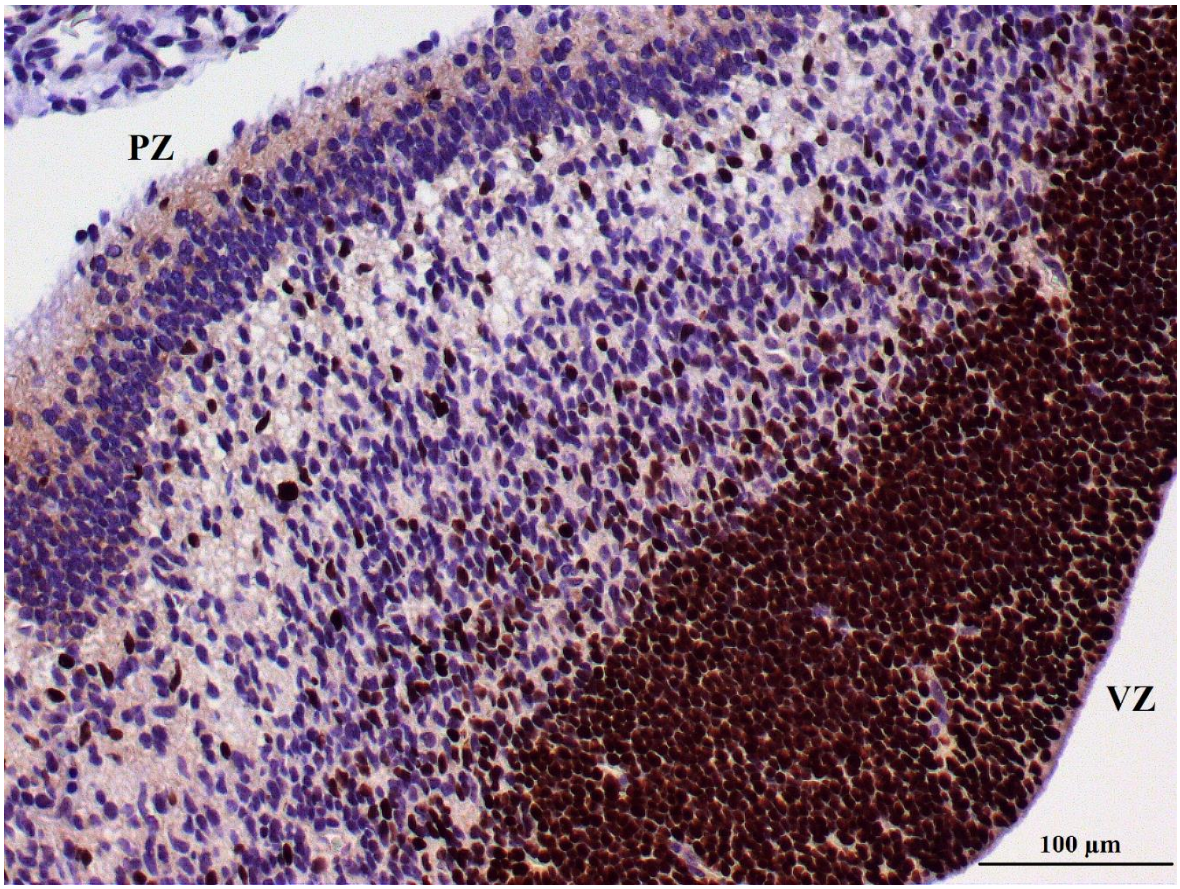

Figure 8. Sox2 immunostaining in the developing cerebral cortex at the 9th week of gestation. The ventricular and subventricular zones show a larger thickness in comparison with the other zones of developing cerebral cortex

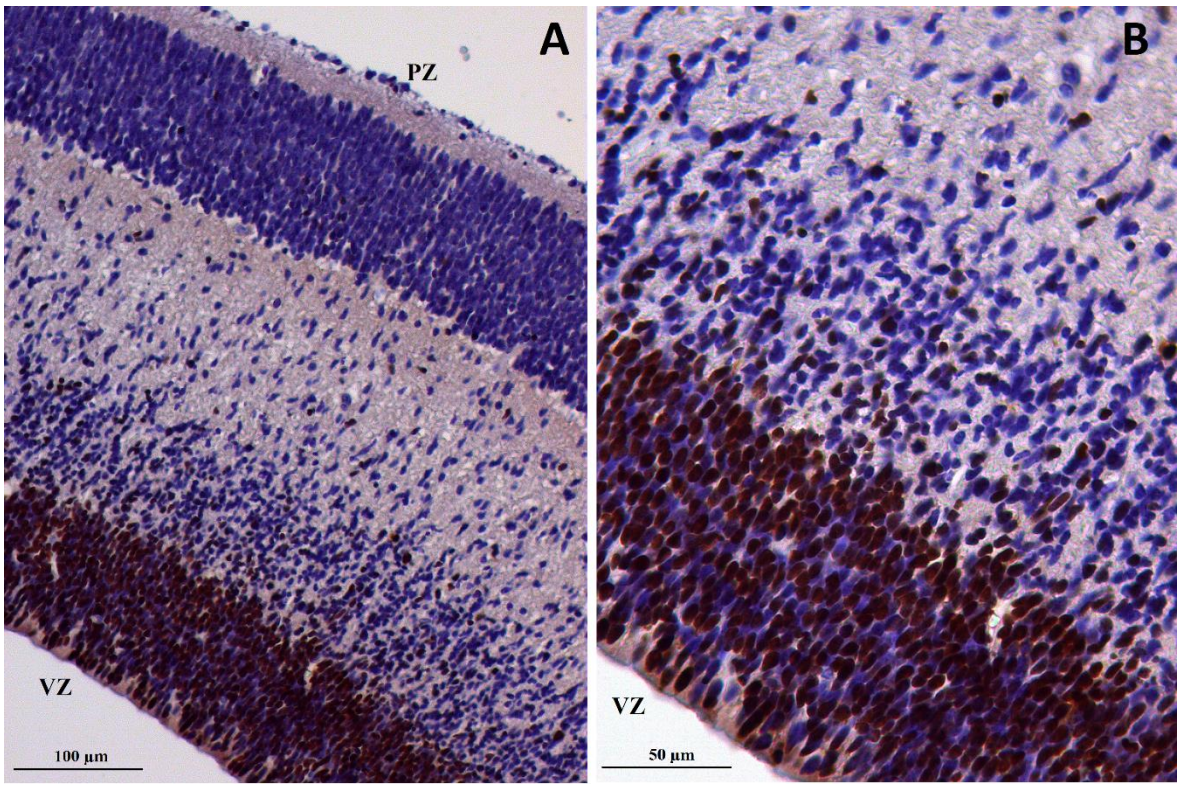

Figure 9. Gestational week 11. A) Sox2 immunoreactivity decreases from the ventricular zone toward the cortical plate; B) Nuclear reactivity of cells localized in the ventricular zone, in the subventricular zone and in migrating newborn neurons

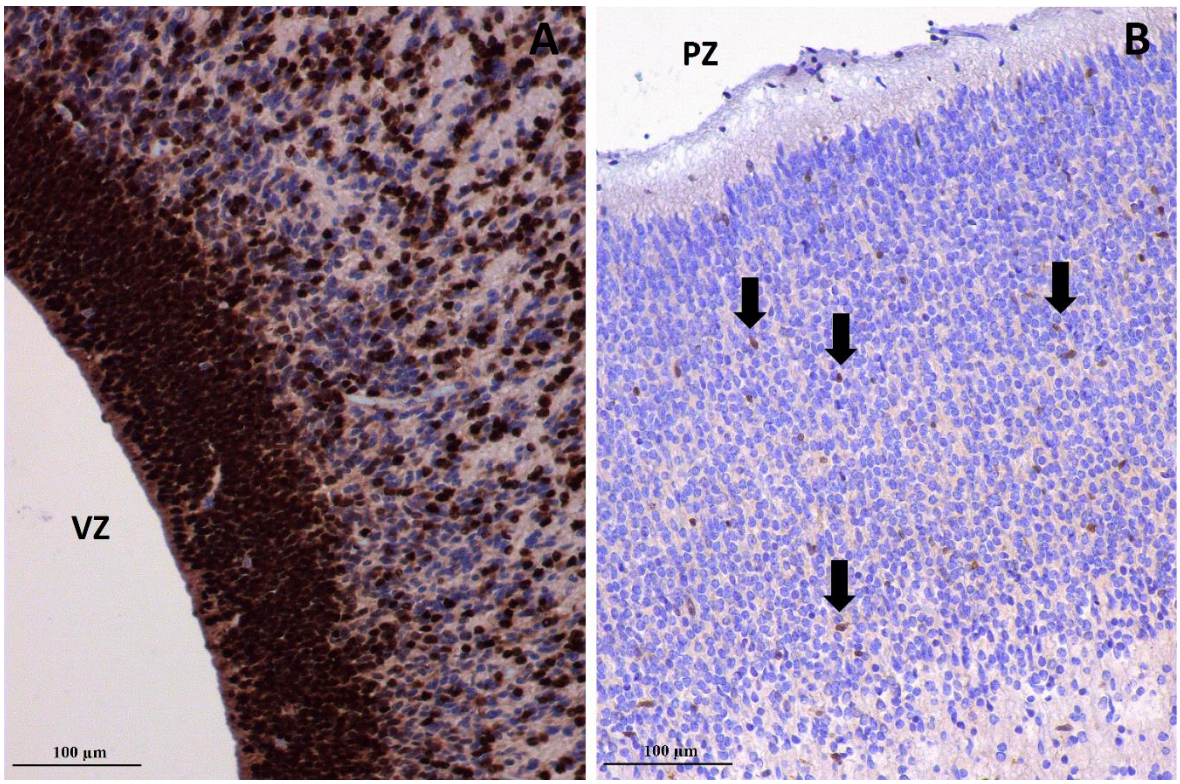

Figure 10. Gestational week 18. A) Sox2 immunoreactivity in ventricular, subventricular and intermediate zones. B) Scattered Sox2-reactive cells in the cortical plate (arrows)

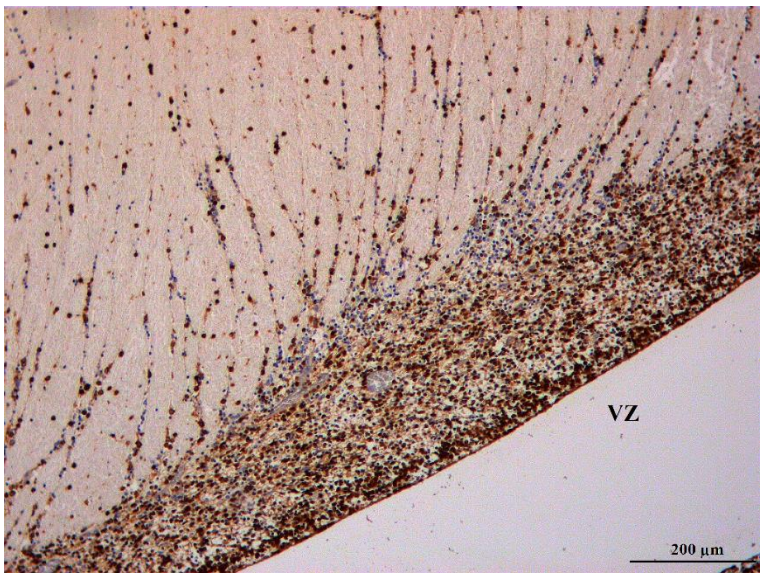

Figure11. Sox2 immunostaining in ventricular and subventricular zones at the 21st week of gestation

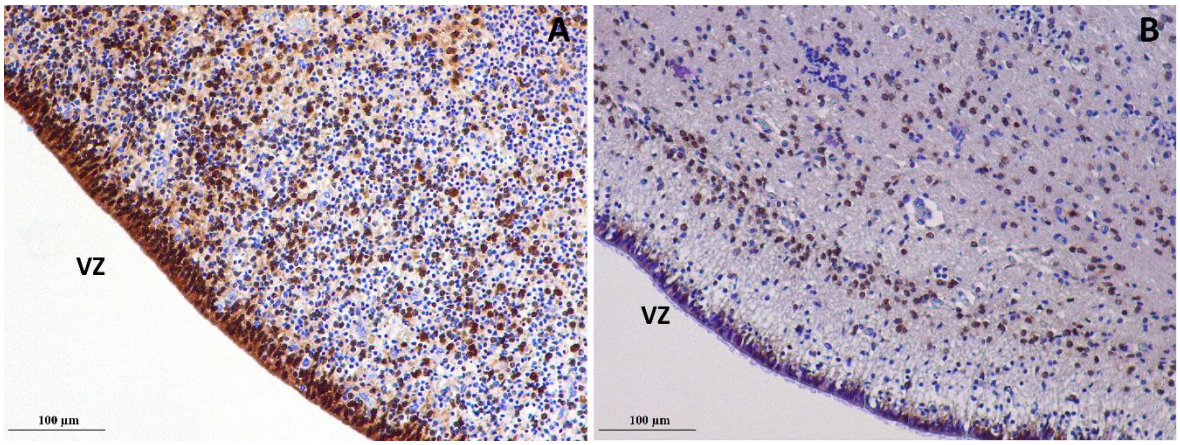

Figure 12. Sox2 immunoreactivity at A) the 34th and at B) the 40th week of gestation

4.1.2 Sox11

At panoramic view, in gestational weeks 9th, 11th and 12th, Sox11 nuclear immunostaining was detected in all cerebral cortex zones (Fig. 13). Sox11 immunoreactivity was particularly strong in subventricular and intermediate zones and in the cortical plate.

From the 15th to the 19th week of gestation, Sox11 nuclear immunoreactivity was particularly strong in subventricular zone, intermediate zone and cortical plate (Fig. 14A). The ventricular zone showed scattered cells Sox11-positive intermingled with no-reactive stem/progenitor cells (Fig. 14B).

After the 21st week until the end of gestation, a mild nuclear Sox11 immunostaining was detected in the cortical layers (Fig. 15A) whereas the ventricular, subventricular and intermediate zones showed scattered Sox11-reactive cells (Fig. 15B).

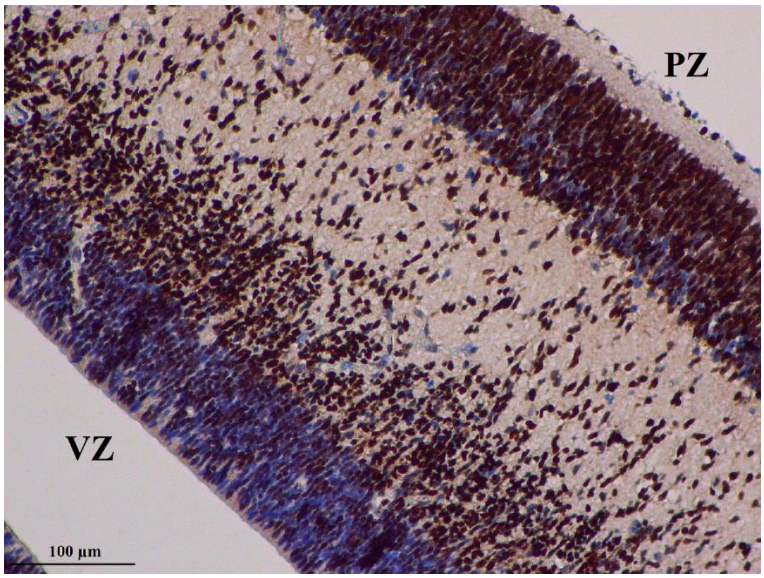

Figure 13. Sox11 immunostaining at the 11th week of gestation

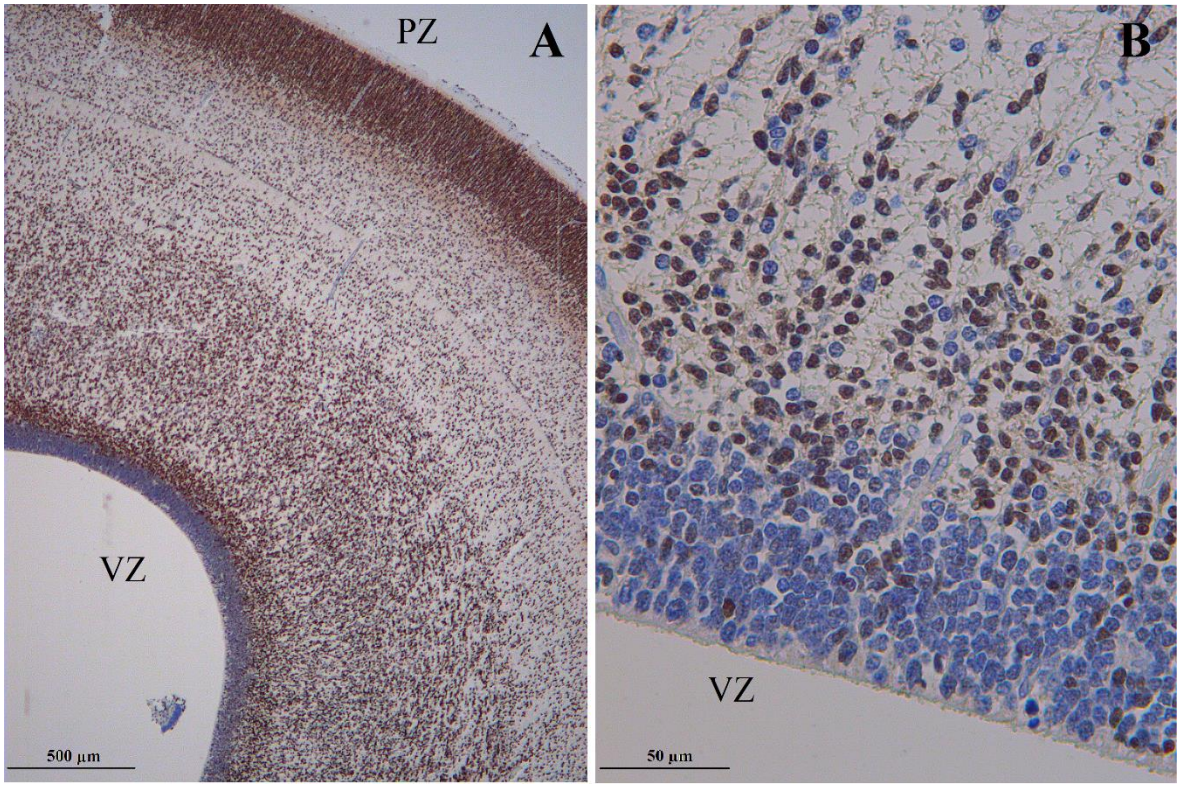

Figure 14. A) Sox11 immunoreactivity at the 17th week of gestation; B) The ventricular, subventricular and intermediate zones at the 18th week of gestation

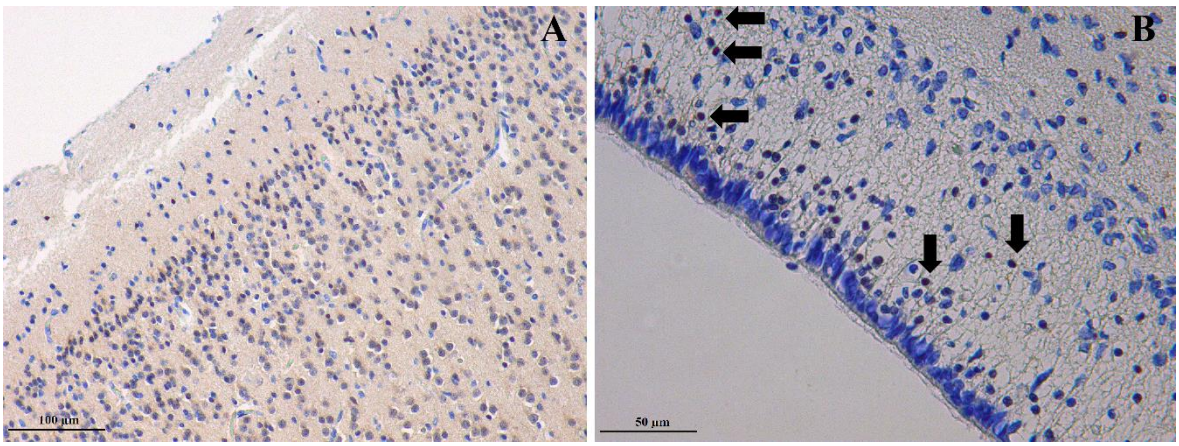

Figure 15. A) Sox11 mild immunoreactivity in cortical layers at the 34 weeks of gestation; B) Scattered Sox11-reactive cells in the ventricular zone at the 39th week of gestation (arrows)

4.1.3 WT1

From the 9th to the 12th week of gestation, WT1 immunostaining was mainly localized in the cytoplasm of radial glia fibers extending from the ventricular zone toward the pial zone (Fig. 16). Nuclei of progenitor cells in both ventricular and subventricular zones did not showed reactivity for WT1 (Fig. 16A). No immunostaining for WT1 was detected in the nuclei of cortical plate neurons, which appeared surrounded by a WT1-positive network formed by the cytoplasmic projection of radial glia cells (Fig. 16B).

From the 15th to the 19th week of gestation, WT1 immunostaing was particularly strong, evidencing the abundance of radial glia fibers extending from the ventricular zone toward the pial zone (Fig. 17).

At 21st week of gestation, WT1 cytoplasmic expression was detected in ventricular and subventricular zones and in radial glia fibers extending toward the pial zone evidencing a decrease in number of radial glia fibers (Fig. 18). Nuclei of both progenitor cells in ventricular and subventricular zones and postmitotic cells of the other zones showed no reactivity for WT1.

At gestational weeks 26 and 30, the radial glial cells in the subcortical layers disappeared. WT1 cytoplasmic immunoreactivity was detected in ependymal cells and in the truncated radial glia fibers in ventricular and subventricular zones (Fig. 19A).

After the 34th week of gestation and until the end of gestation, a weak WT1 reactivity was detected in ependymal cells (Fig 19B). Endothelial cells of small blood vessels showed a strong immunostaining for WT1 in all gestational ages of the developing cerebral cortex.

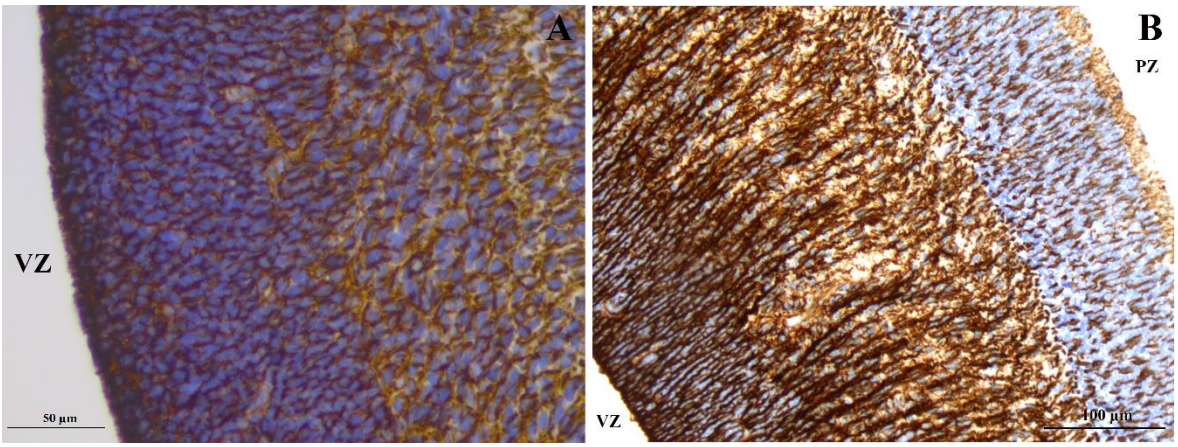

Figure 16. A) WT1 immonoreactivity in ventricular and subventricular zones at the 9th week of gestation; B) Immunoreactivity for WT1 at the 11th week of gestation in radial glia cells extending from the ventricular zone toward the pial zone

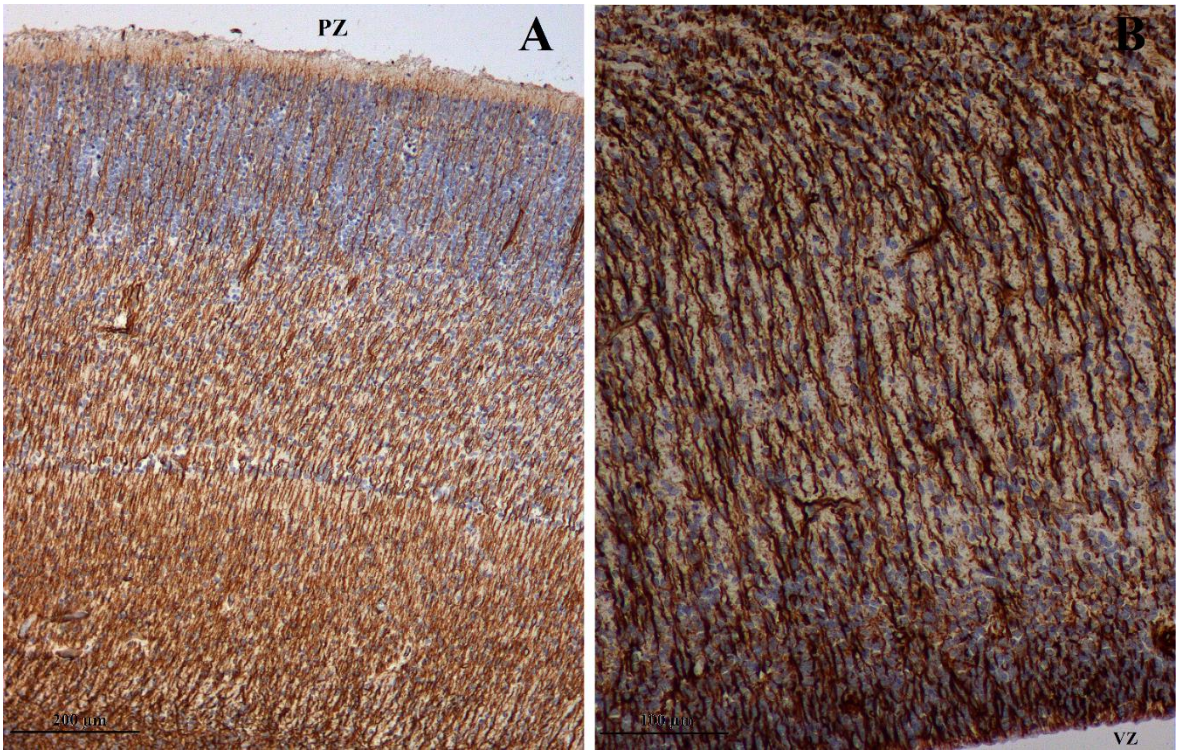

Figure 17. At the 17th week of gestation, WT1 immunoreactivity evidences an increase of radial glia fibers in the intermediate zone and in the cortical plate (A), and also in the ventricular and subventricular zones (B)

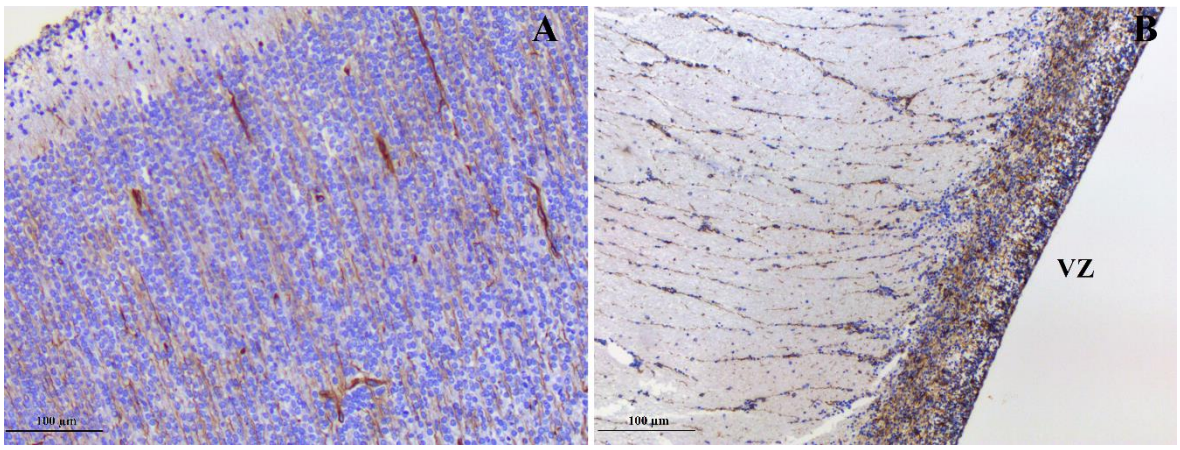

Figure 18. WT1 immunoreactivity at the 21st week of gestation showed a decrease of radial glia fibers in the cortical plate (A) and in the ventricular, subventricular and intermediate zones (B)

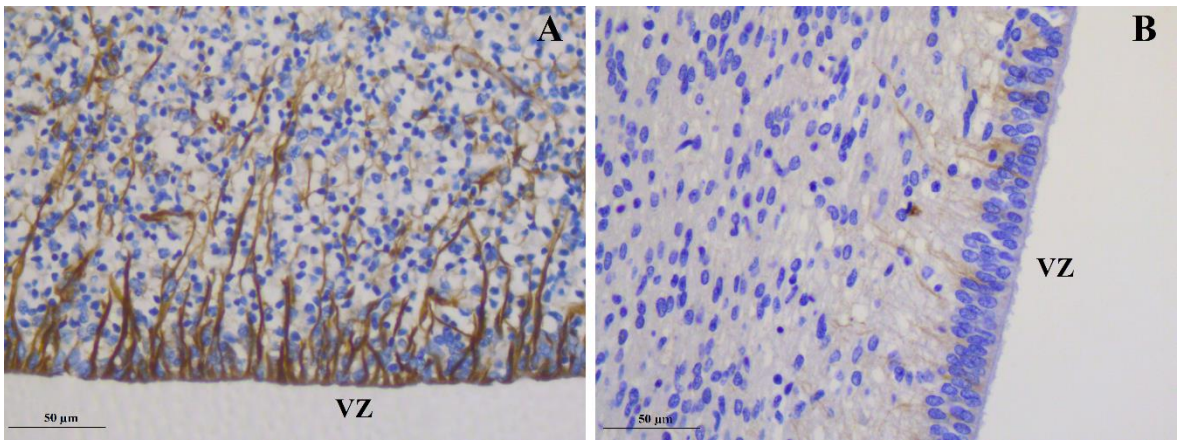

Figure 19. A) WT1 cytoplasmic immunoreactivity in ependymal cells and in the truncated radial glia fibers in ventricular and subventricular zone at gestational week 30; B) Mild immunostaining for WT1 in ependymal cells at the 39th week of gestation

4.1.4 Nestin

From the 9th to the 12th week of gestation, a strong Nestin cytoplasmic immunoreactivity was detected in the ventricular, subventricular and intermediate zones, associated with a weak immunostaining in the cortical plate and pial zone (Fig. 20A). At higher magnification, immunostaining for Nestin was detected in the projection of radial glia cells extending from the ventricular zone toward the cortical plate (Fig. 20B).

From the 15th to the 21th week of gestation, the radial glia fibers increase in number. At these gestational ages, Nestin cytoplasmic immunoreactivity was detected in radial glia fibers extending from ventricular zone toward pial zone (Fig 21A). At this time point, Nestin

immunoreactivity was stronger in cortical plate in comparison to the previously gestational weeks (Fig. 21B). No reactivity for Nestin was found in nuclei of stem/progenitor cells in both ventricular and subventricular zones and in postmitotic cells of the other zones.

Between the 24th and the 30th gestational week, the radial glial fibers in the subcortical layers disappeared. Accordingly, cytoplasmic immunostaining for Nestin was detected solely in ependymal cells and in truncated radial glia fibers of the ventricular zone (Fig. 22). No reactivity for Nestin was found in the others cerebral cortex zones, being express only in endothelial cells of blood vessels.

At the end of gestation, Nestin showed no reactivity in both neuron and glial cells of grey and white matter.

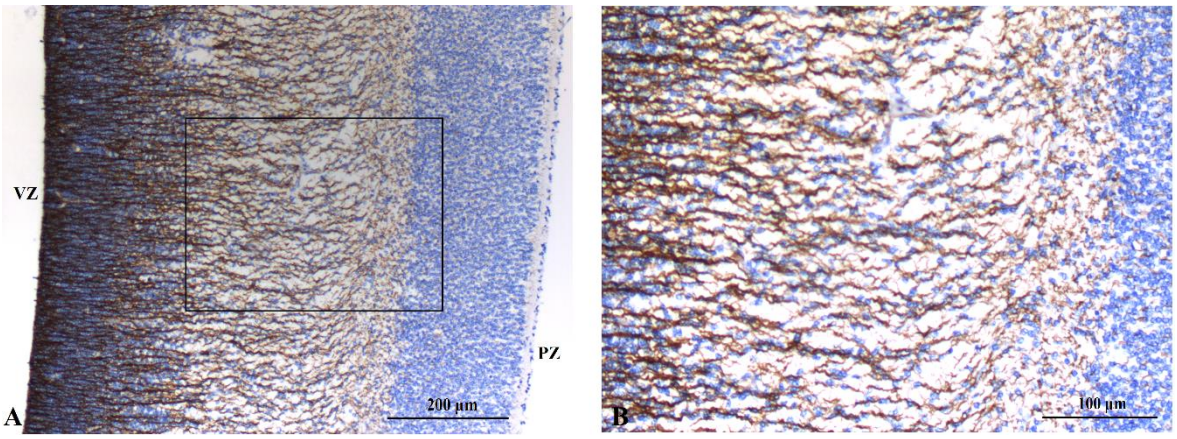

Figure 20. Gestational week 11; A) Nestin immunoreactivity in the ventricular, subventricular and intermediate zones; B) Positivity of radial glia cells extending from the ventricular zone toward the cortical plate

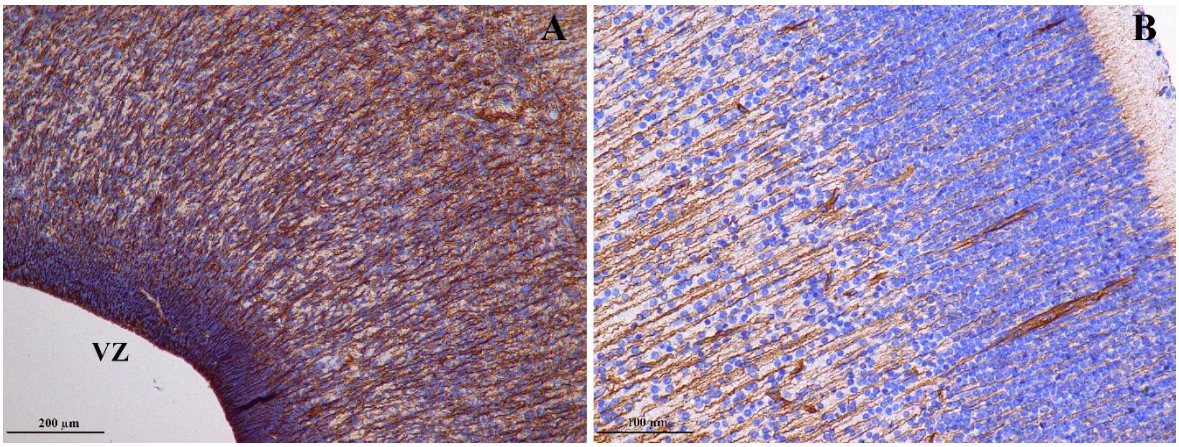

Figure 21. At gestational week 17, Nestin immunoreactivity evidenced the increase of radial glia fibers in the ventricular, subventricular, intermediate zones (A) and in the cortical plate (B)

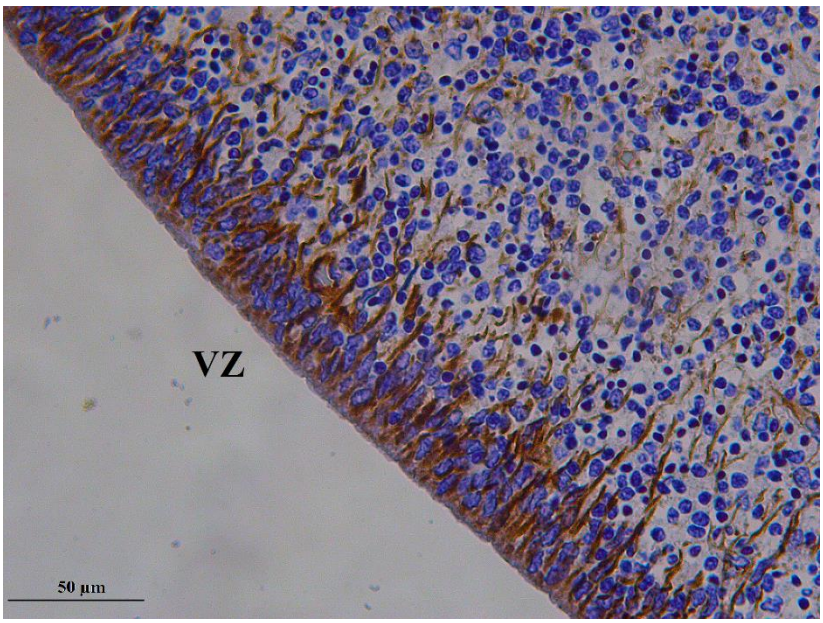

Figure 22. Immunoreactivity for Nestin in ependymal cells at gestational week 30

4.1.5 Vimentin

From the 9th to the 12th week of gestation, Vimentin immunoreactivity was detected in all the developing cerebral cortex zones being restricted to cytoplasmic projection of radial glia cells extending from the ventricular zone toward the cortical plate (Fig. 23A). No immunostaining for Vimentin was detected in nuclei of the radial glia cells, intermediate progenitor cells and in postmitotic cells (Fig. 23B).

From the 15th to the 21st week of gestation, immunostaining for Vimentin evidenced an increase of radial glia fibers extending from the ventricular zone toward the pial zone (Fig 24).

After 24 weeks until the end of gestation, Vimentin immunoreactivity was detected in ependymal cells and in truncated radial glia fibers of ventricular zone (Fig. 25). Moreover, immunostaining for Vimentin was found in endothelial cells both in grey and white matter. At these gestational ages, no reactivity for Vimentin was found in mature neurons of the cerebral cortex layers.

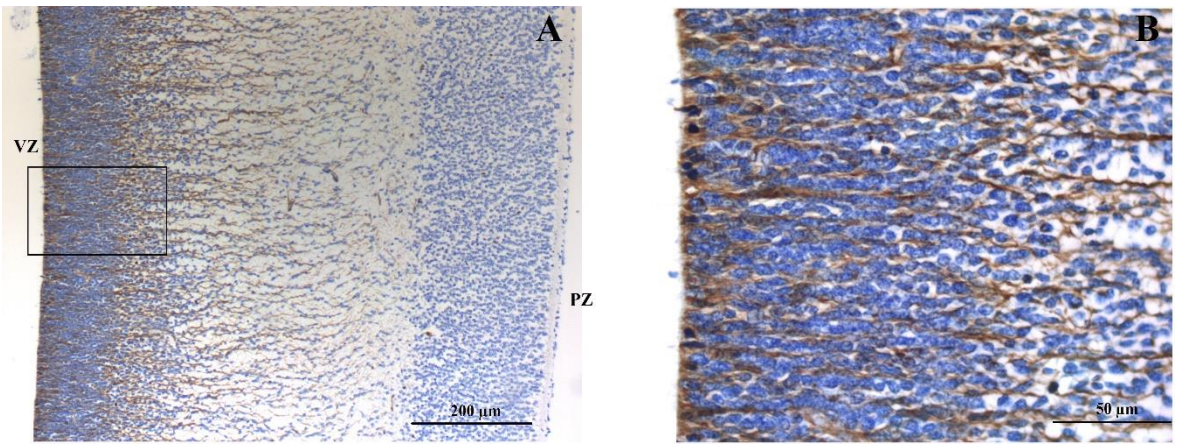

Figure 23. 11th week of gestation. A) Vimentin immunoreactivity of radial glia cells extending from the ventricular zone toward the cortical plate; B) No reactivity was detected in nuclei in both the ventricular and subventricular zones

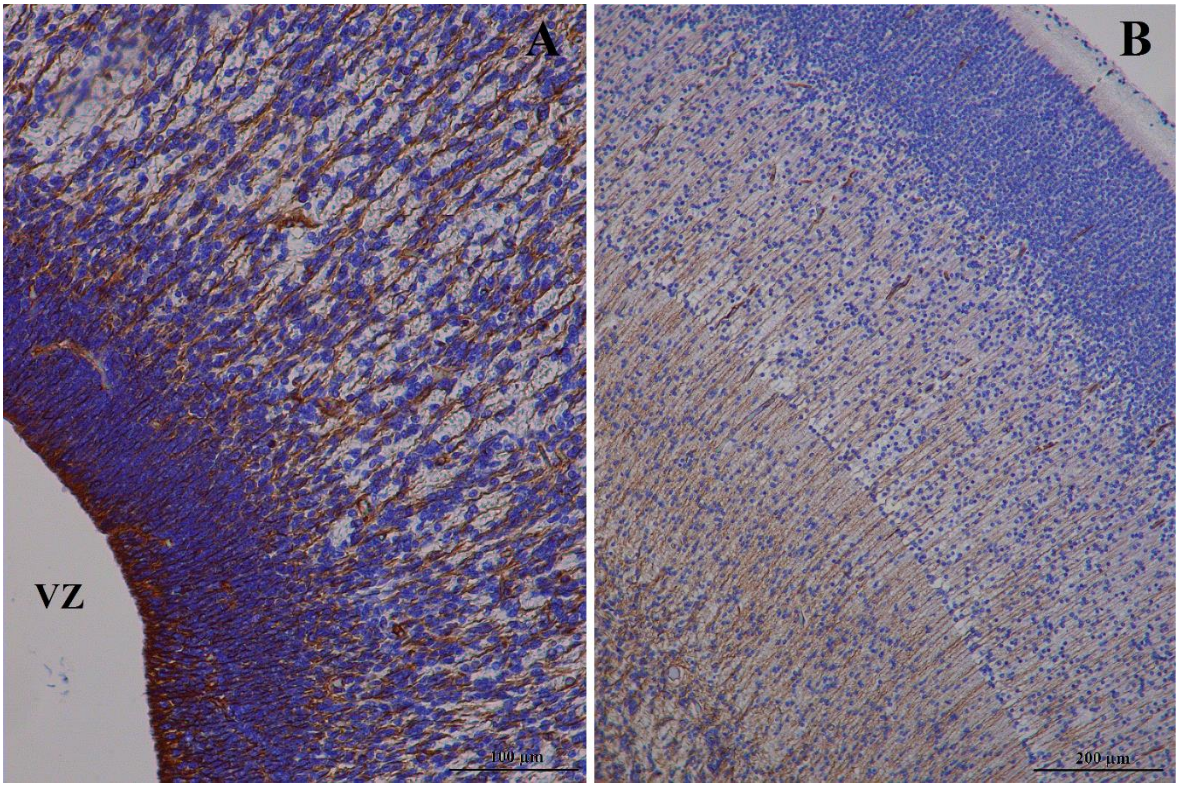

Figure 24. Gestational week 17. Immunostaining for Vimentin evidenced the increase of radial glia fibers in the ventricular and subventricular zones (A), and in the intermediate zone and the cortical plate (B)

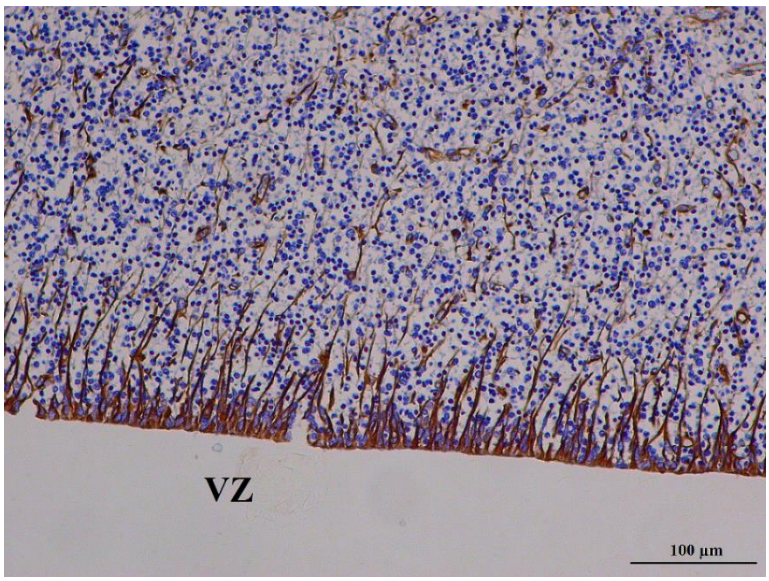

Figure 25. Vimentin immunostaining in ependymal cells and in truncated radial glia fibers of ventricular zone at gestational week 30

4.1.6 Pax2

From the 9th to the 12th week of gestation, Pax2 nuclear reactivity progressively increased from the ventricular zone toward the pial zone (Fig. 26A). The cortical plate and the intermediate zone showed several cells with nuclear reactivity for Pax2 intermingled with no-reactive cells. At higher power, the vast majority of subpial zone cells showed a strong reactivity for Pax2 (Fig. 26B). Scattered Pax2-reactive cells were also detected into the pial zone.

From the 15th to the 18th week of gestation, an increase of Pax2-reactive cells was detected in all the cerebral cortex zones especially in the intermediate zone and in the cortical plate (Fig. 27).

At gestational week 21, nuclear immunoreactivity for Pax2 decreased in all cerebral cortex zones (Fig. 28). The cortical plate showed a higher number of no-reactive cells for Pax2 than the reactive ones (Fig. 28A).

At the 24th week of gestation, Pax2 immunostaining was detected in ventricular zone, being expressed in scattered cells with high reactivity intermingled with mild-reactive cells and no-reactive cells (Fig. 29).

At the 30th week of gestation, immunoreactivity for Pax2 was restricted to ependymal cells and scattered cells in the others cerebral cortex zones (Fig. 30A).

After the 34th week until the end of gestation, Pax2 showed a mild immunoreactivity in scattered cells in the cerebral cortex layers (Fig. 30B).

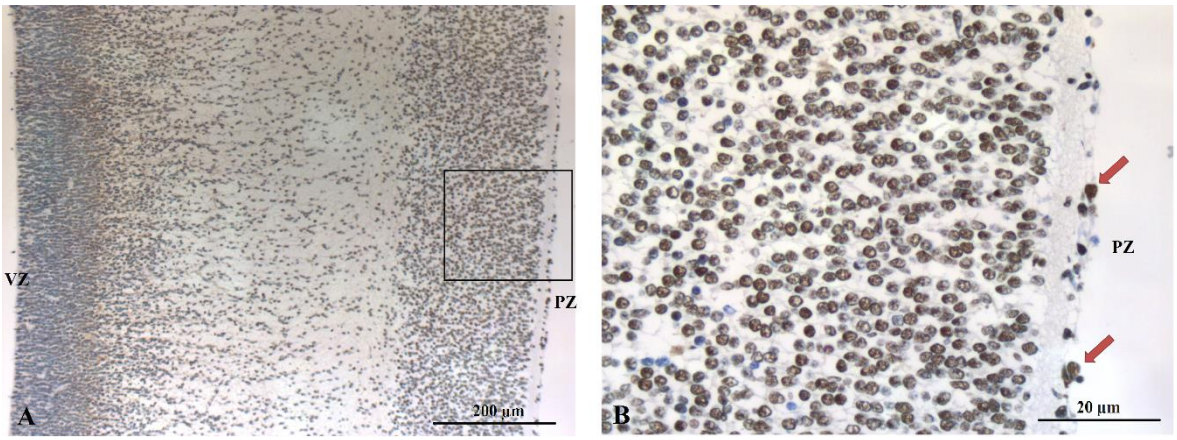

Figure 26. Gestational week 11; A) Pax2 nuclear reactivity progressively increases from the ventricular zone toward the pial zone; B) Immunoreactivity of the cortical plate cells intermingled with no-reactive cells and scattered positive cells in the pial zone (arrows)

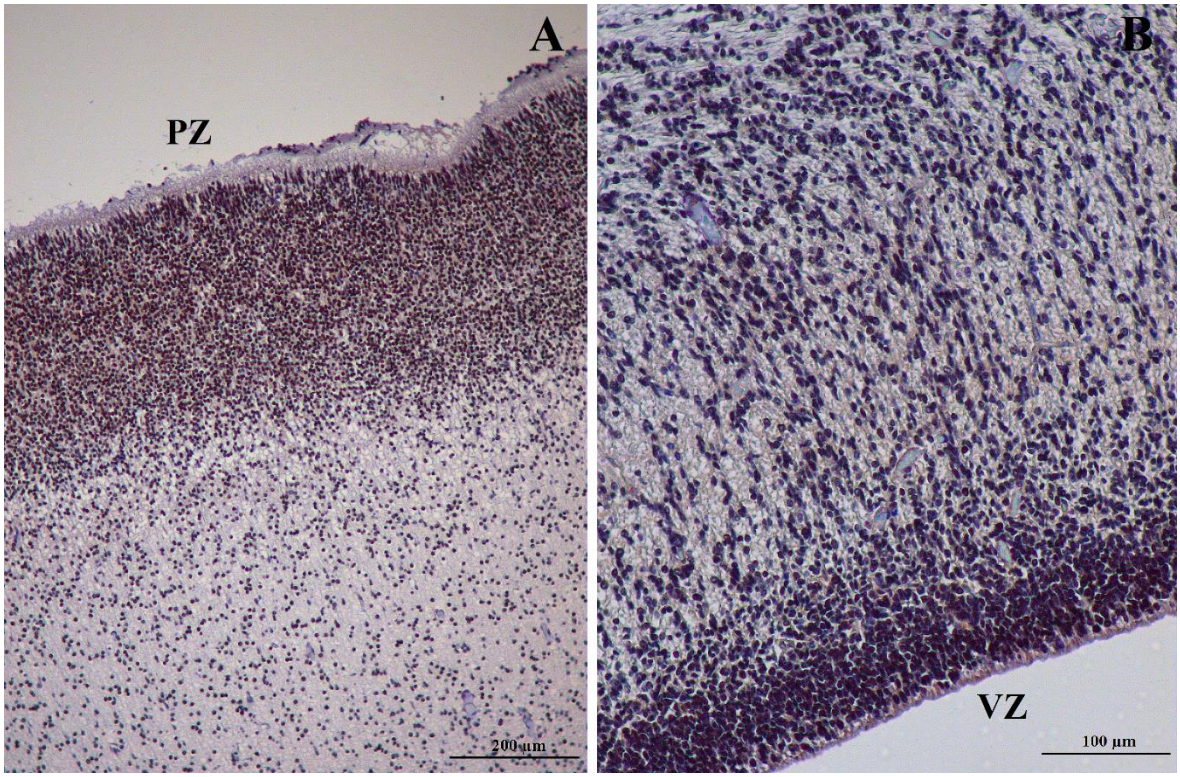

Figure 27. Strong Pax2 immunoreactivity in cortical plate and intermediate zone (A) and in ventricular and subventricular zones (B)

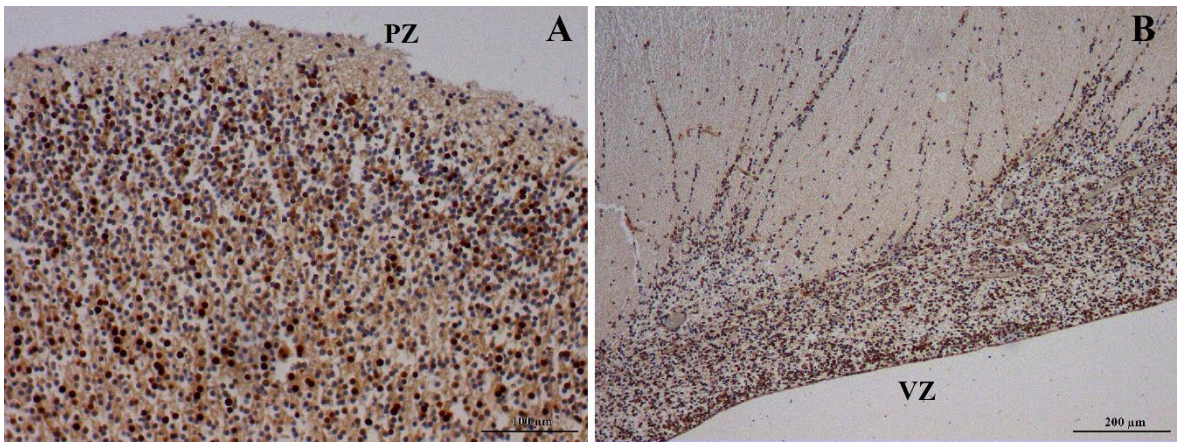

Figure 28. Gestational week 21. A) the cortical plate showed a higher number of no-reactive Pax2 cells as compared to the reactive ones; B) Pax2 immunoreactivity decrease in ventricular, subventricular and intermediate zones

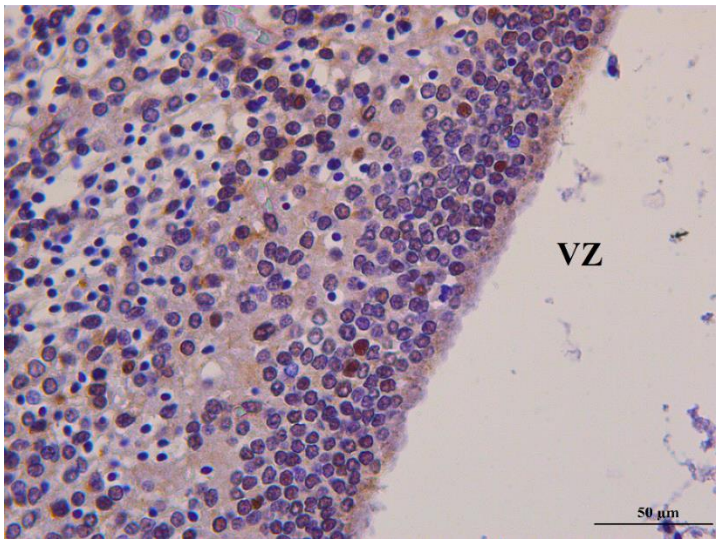

Figure 29. At the 24th week of gestation scattered Pax2-reactive cells were detected in the ventricular zone

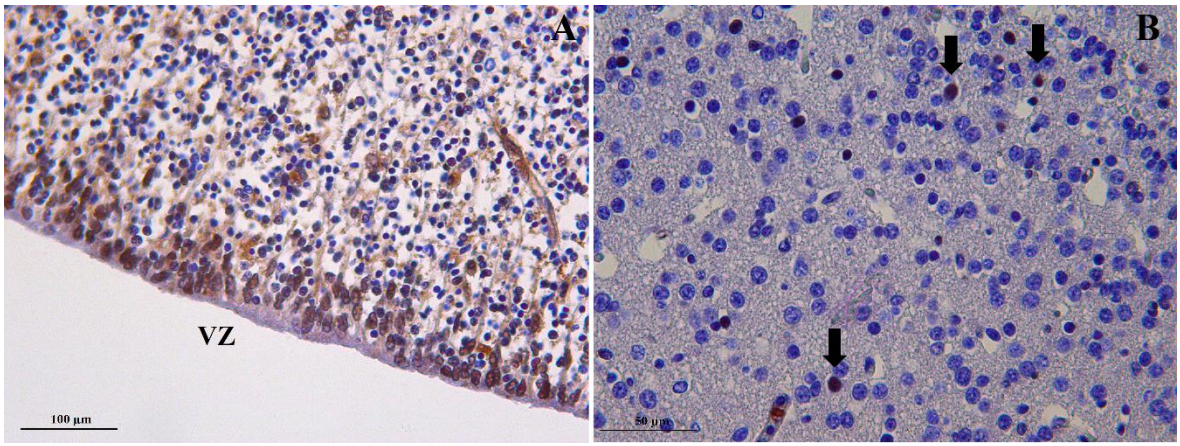

Figure 30. A) Pax2 immunoreactivity in ependymal cells and scattered cells of ventricular zone at gestational week 30; B) Mild immunoreactivity for Pax2 in scattered cells of the cerebral cortex layers (arrows)

4.1.7 Pax6

At gestational week 9, Pax6 was highly expressed in stem/progenitor cells in the ventricular and subventricular zones (Fig. 31). A mild nuclear immunoreactivity for Pax6 was detected in scattered cells of intermediate zone. No immunoreactivity was found in cortical plate and in pial zone.

From the 11th to the 17th week of gestation, nuclear immunostaining for Pax6 decreased in intensity in ventricular and subventricular zones. The ventricular zone showed a stronger immunoreactivity for Pax6 compared to the subventricular zone (Fig. 32). Scattered Pax6-reactive cells was also found in intermediate zone at gestational week 17 (Fig. 32B). The postmitotic cells of intermediate zone, cortical plate and pial zone showed no immunoreactivity for Pax6.

At the 21st weeks of gestation, nuclear immunoreactivity for Pax6 was detected in scattered cells mainly localized in the ventricular and subventricular zones (Fig. 33).

No immunoreactivity for Pax6 was found after the 24th week until the end of gestation.

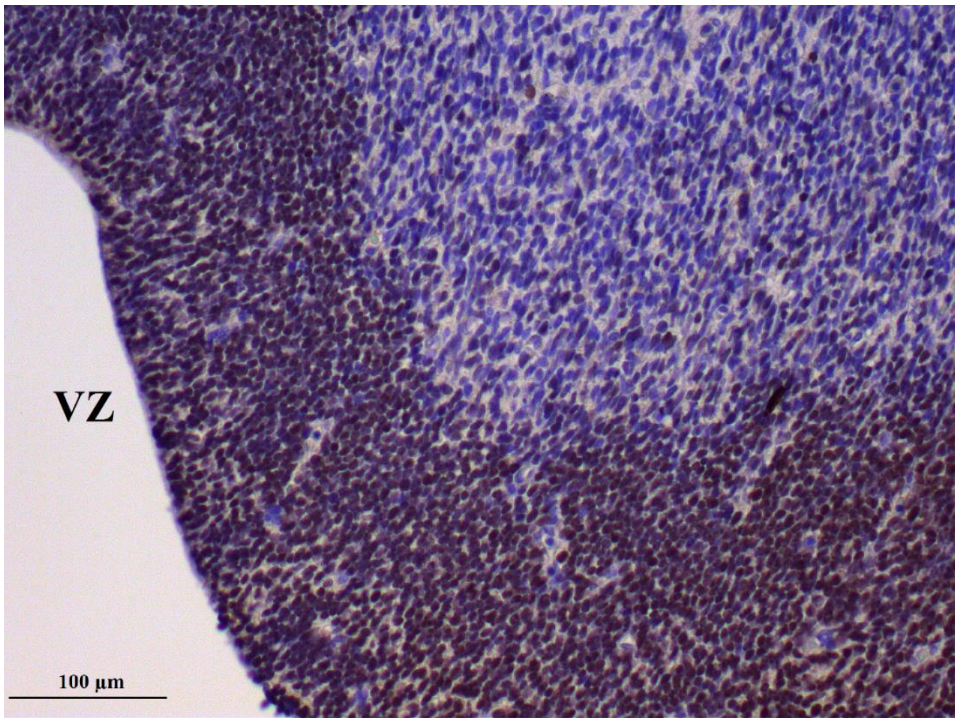

Figure 31. Pax6 immunoreativity in ventricular and subventricular zones at gestational week 9

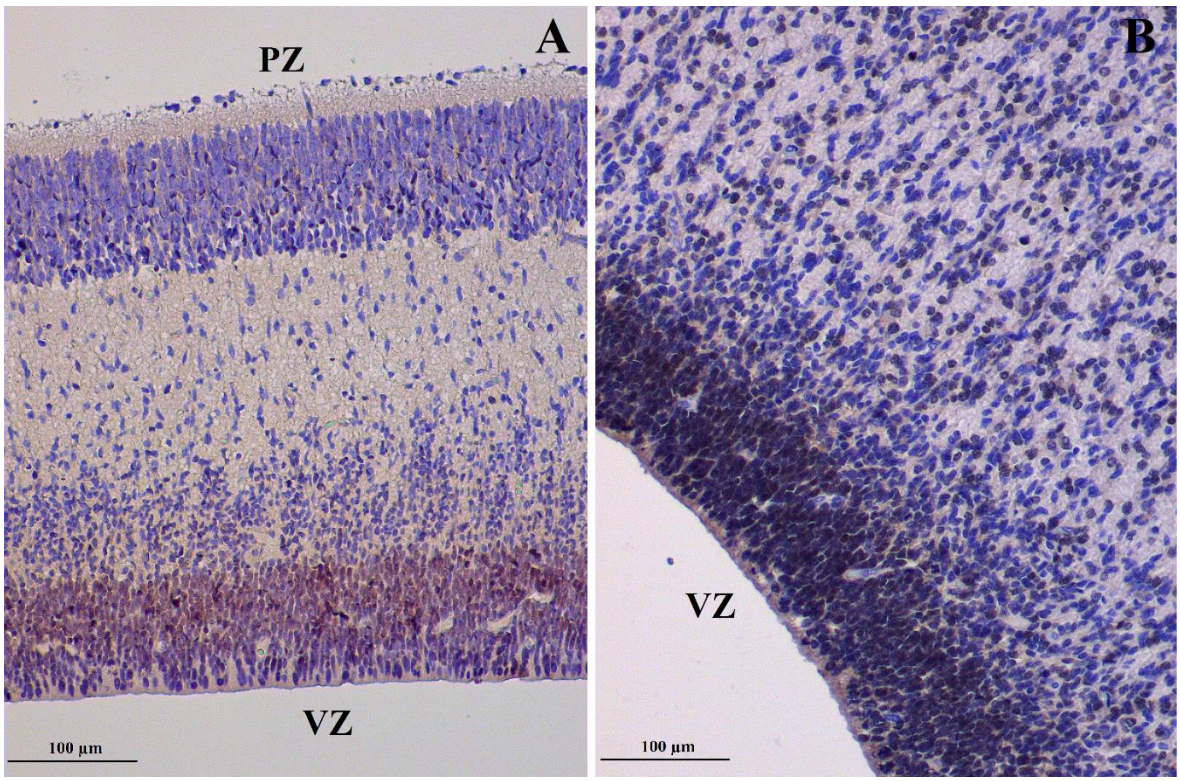

Figure 32. Pax6 immunoreactivity in ventricular zone at gestational week 10 (A) and in gestational week 17 which showed scattered Pax6-reactive cells in intermediate zone (B)

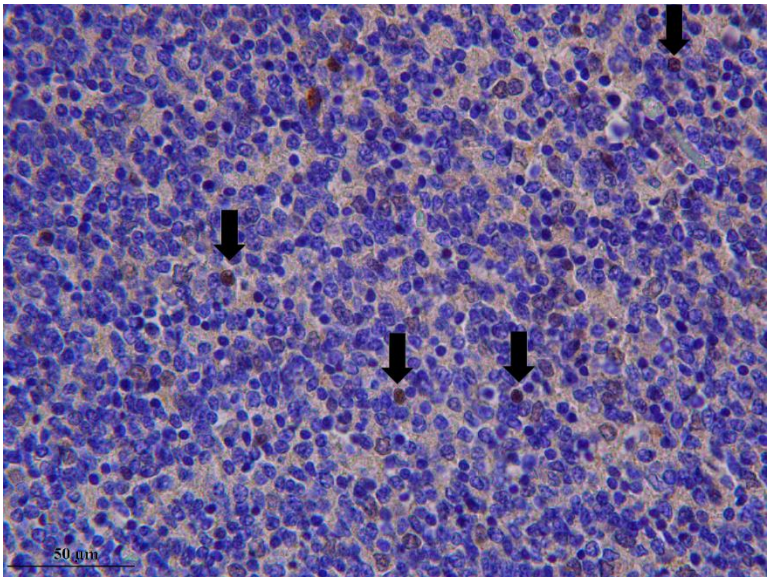

Figure 33. Scattered Pax6-reactive cells in the ventricular and subventricular zones at gestational week 21 (arrows)

4.2 Mature neurons markers

4.2.1 Neurofilament

From the 9th to the 26th weeks of gestation, no immunoreactivity for Neurofilament was found in all the developing cerebral cortex zones.

After 30 weeks until the end of gestation, a strong nuclear and cytoplasmic immunostaining for Neurofilament was detected in mature neurons (Fig. 34). No reactivity for Neurofilament was found in glial cells and the ventricular zone including ependymal cells (Fig. 35).

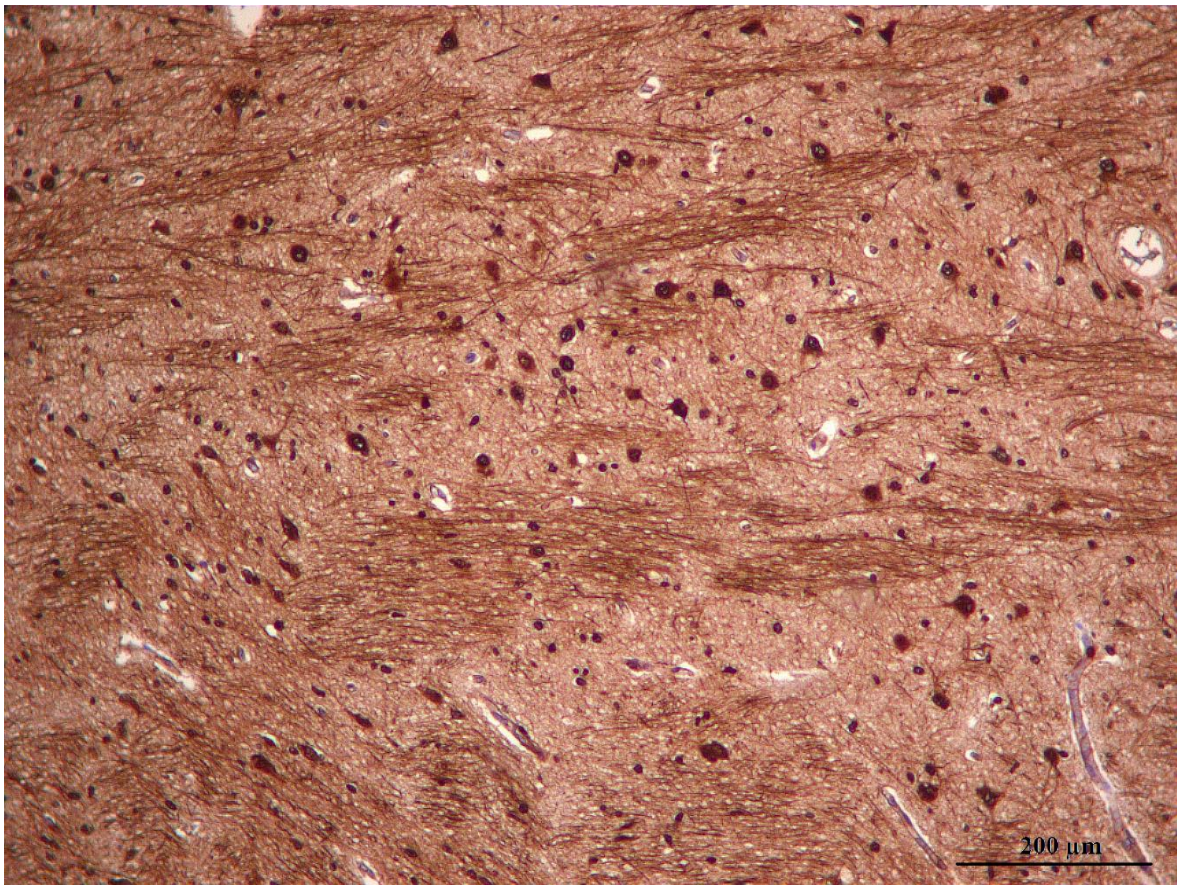

Figure 34. Strong nuclear and cytoplasmic immunoreactivity for Neurofilament at gestational week 34

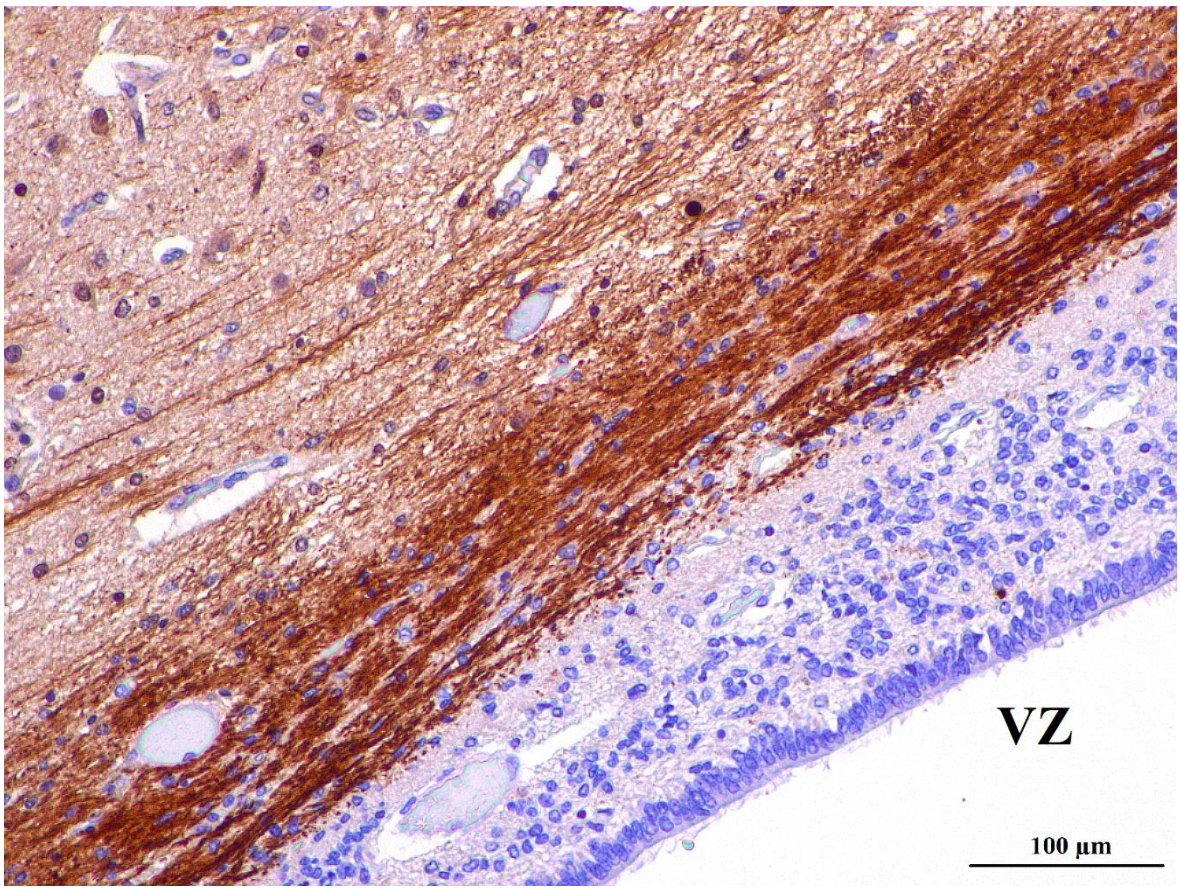

Figure 35. Neurofilament cytoplasmic immunoreactivity at gestational week 30

4.2.2 NSE

From the 9th to the 12th week of gestation, at lower power, immunostaining for NSE was detected in the intermediate zone, in the cortical plate and in the pial zone (Figure 36A). At higher magnification, an intense expression of NSE was detected in the cytoplasm of cells localized in the intermediate zone and in the cortical plate (Fig. 36B). A mild expression for NSE was also found in the subventricular zone in the absence of immunoreactivity in the ventricular zone.

From the 15th to the 21th week of gestation, cytoplasmic and nuclear immunoreactivity of NSE increased in intermediate zone, cortical plate and pial zone (Fig. 37A). No significant staining was detected in both ventricular and subventricular zones (Fig. 37B).

After 24 weeks until the end gestation, the NSE cytoplasmic and nuclear immunoreactivity increased both in grey and white matter, in the absence of any reactivity in ependymal cells of ventricular zone.

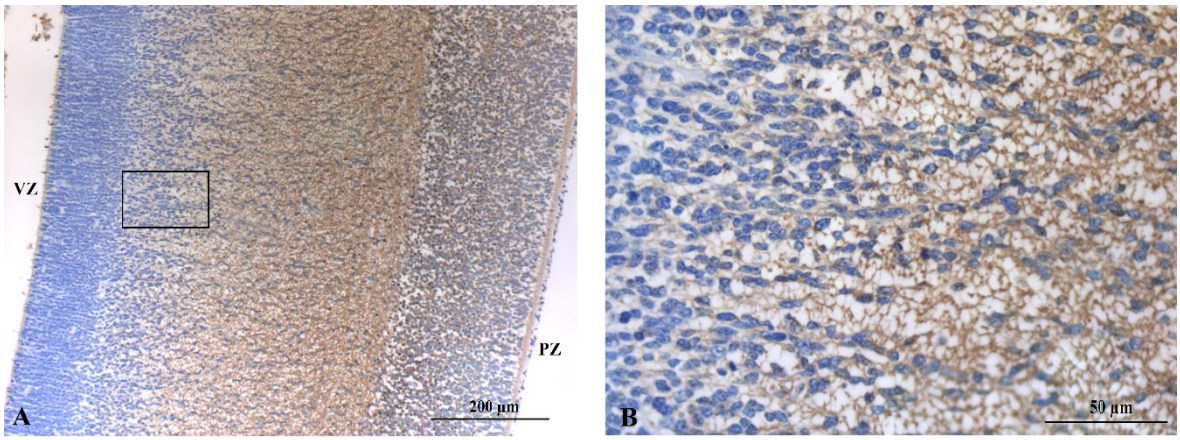

Figure 36. Gestational week 11. A) NSE immunostaining in the intermediate zone, in the cortical plate and in the pial zone; B) Cytoplasmic positivity in the intermediate zone cells in contrast to non-reactive cells of subventricular zone

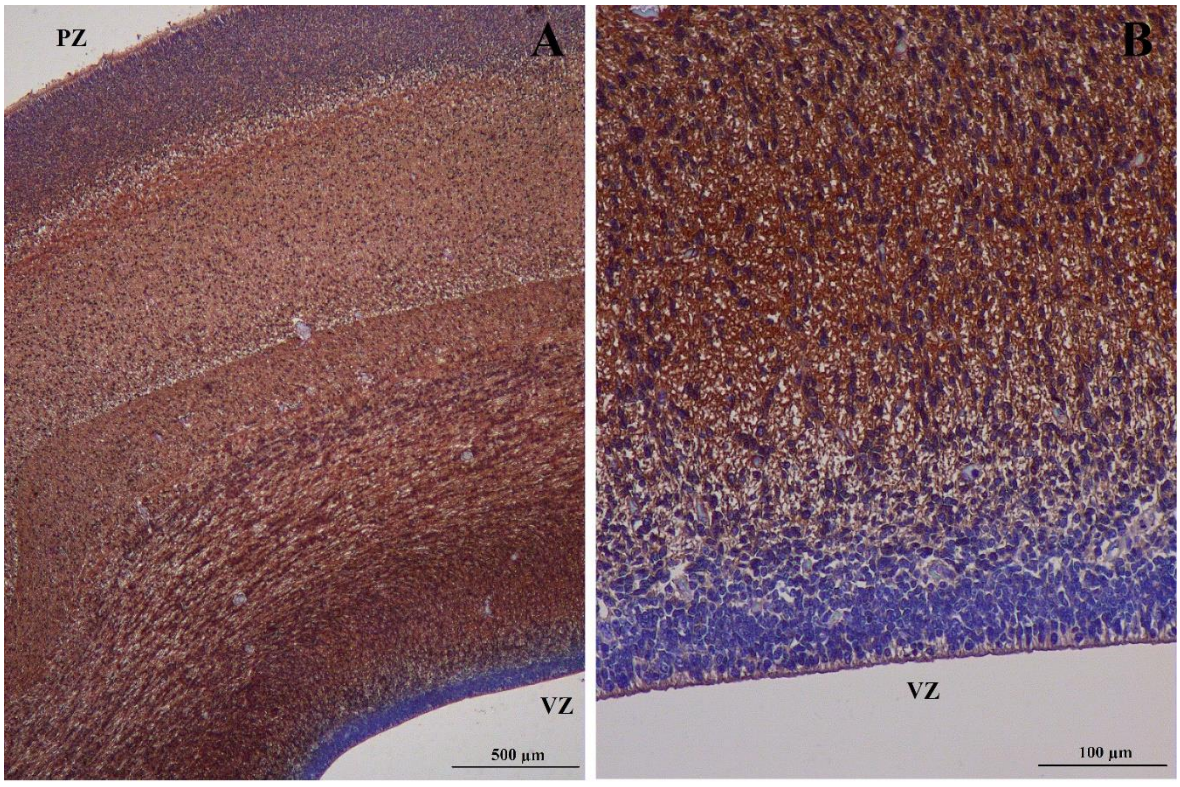

Figure 37: Increasing of NSE immunoreactivity in intermediate zone, cortical plate and pial zone at gestational week 17 (A). no reactivity for NSE in ventricular and subventricular zones (B)

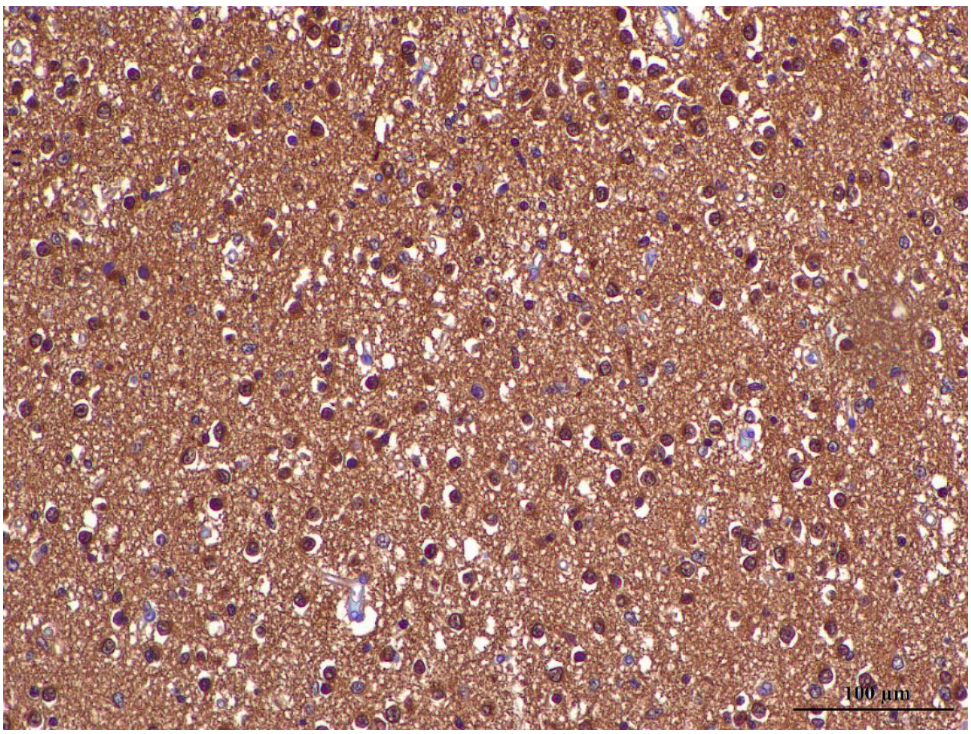

Figure 38. NSE cytoplasmic and nuclear immunostaining at gestational week 34

4.2.3 Synaptophysin

Between the 9th and the 12th week of gestation, no immunoreactivity for Synaptophysin was found in all the developing cerebral cortex zones.

From the 15th to the 21st week of gestation, Synaptophysin was highly expressed in cytoplasm of cells localized in the intermediate, subplate and subpial zones. A mild cytoplasmic reactivity was also found in the cortical plate (Fig. 39A). No immunoreactivity for Synaptophisyn was detected in stem/progenitor cells of the ventricular and subventricular zones (Fig. 39B).

After the 24th week until the end of gestation, a strong cytoplasmic immunoreactivity for Synaptophysin was detected both in grey and white matter in the absence of any reactivity in ependymal cells (Fig. 40).

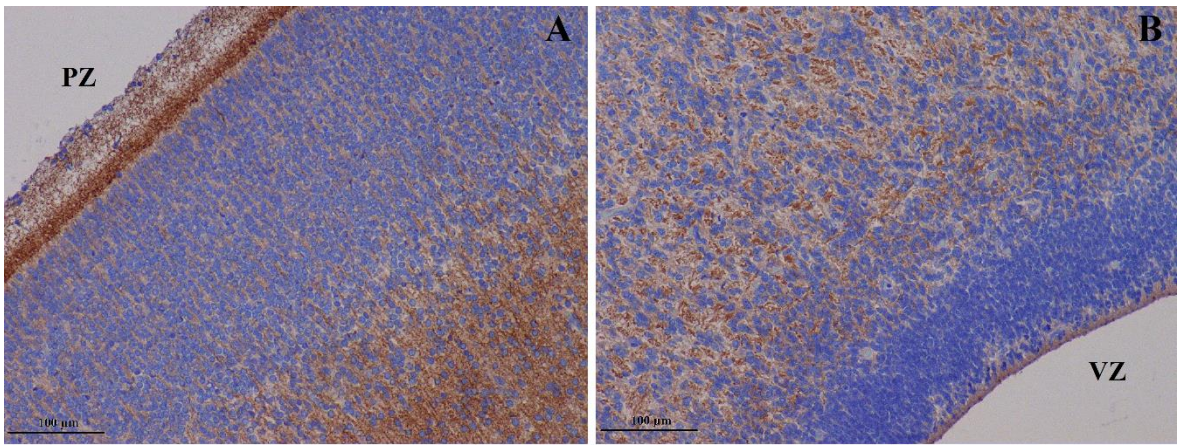

Figure 39. Gestational week 15. A) Synaptophysin immunoreactivity at in the intermediate, subplate and subpial zones associated whit a mild cytoplasmic reactivity in the cortical plate; B) no reactivity was found in ventricular and subventricular zones

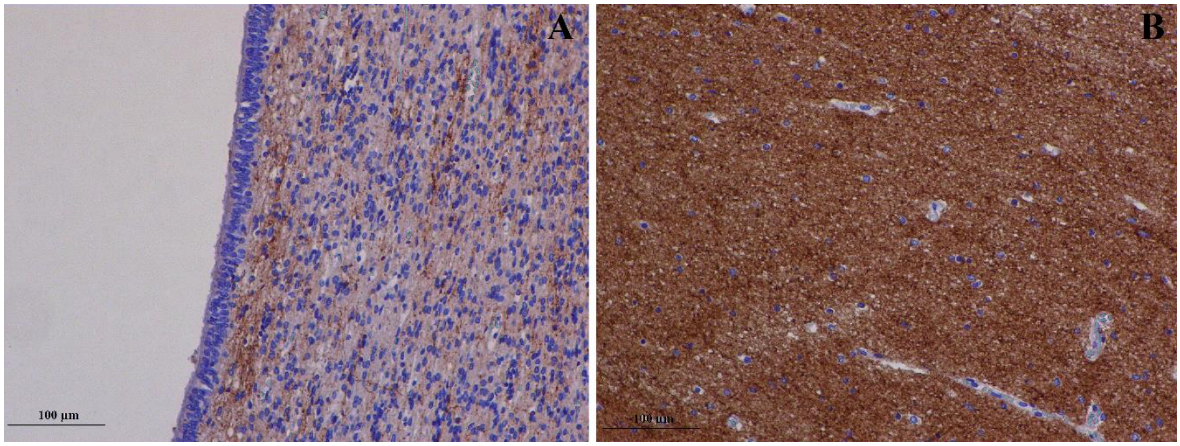

Figure 40: Immunostaining for Synaptophysin at gestational week 34 showed A) no reactivity in ependymal cells in contrast with B) a strong cytoplasmic immunoreactivity in the others zones

4.3 Mature glia markers

4.3.1 GFAP

Between the 9th and the 18th week of gestation, no reactivity for GFAP was detected in all the cerebral cortex zones.

At 21 and 24 weeks of gestation, intense GFAP cytoplasmic immunostaining was detected in the ventricular zone and in scattered cells subventricular and intermediate zones (Fig. 41). No reactivity was found in the cortical plate.

At gestational week 30, GPAP expression was detected in the ventricular zone including ependymal cells and truncated radial glia fibers (Fig. 42A). Moreover, a strong nuclear and cytoplasmic GFAP immunostaining was detected in glial cells of intermediate zone in the absence of reactivity in mature neurons (Fig. 42B). In subpial layers, GFAP showed a lesser intensity of immunoreactivity.

From the 34th week until the end of gestation, a strong nuclear and cytoplasmic expression of GFAP was detected in mature glial cells both in grey and white matter in the absence of any reactivity in mature neurons (Fig. 43).

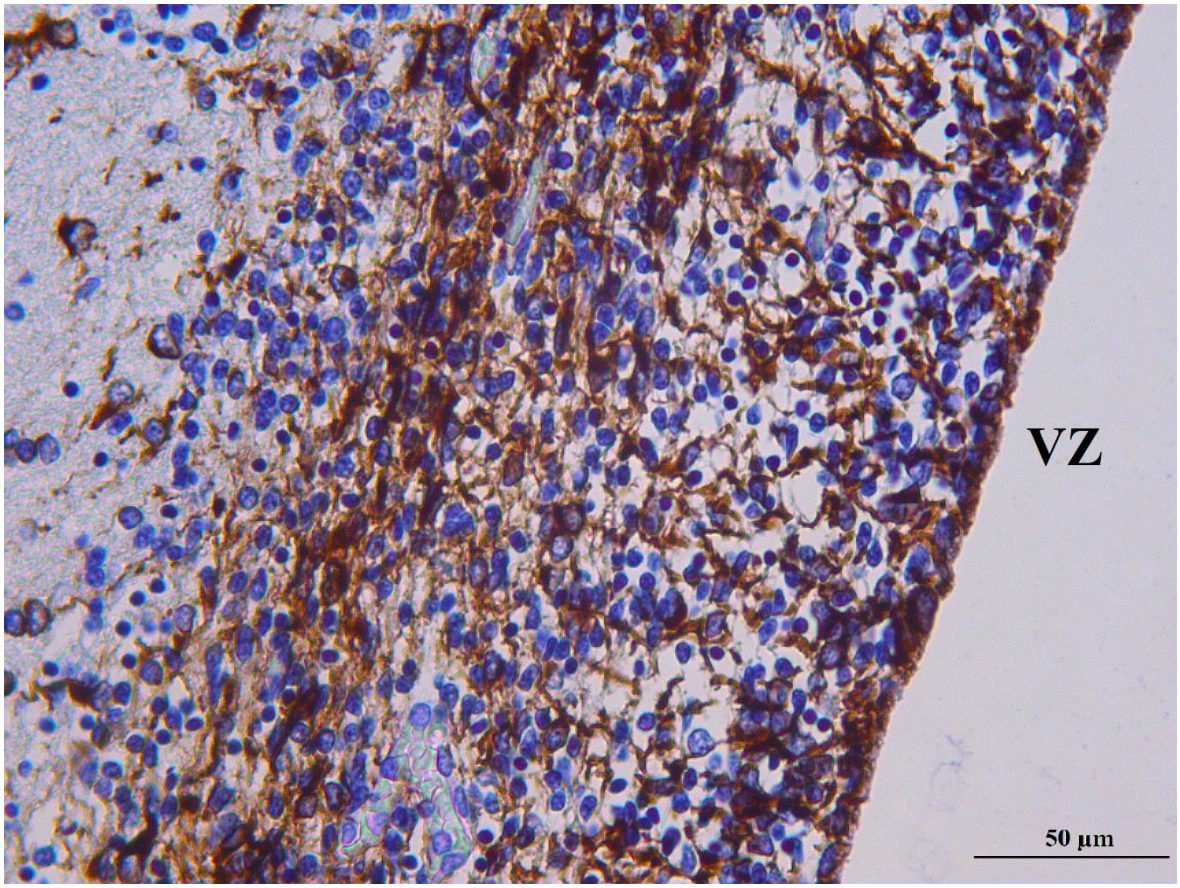

Figure 41. GFAP cytoplasmic immunostaining in the ventricular zone and in scattered cells subventricular and intermediate zones at gestational week 21

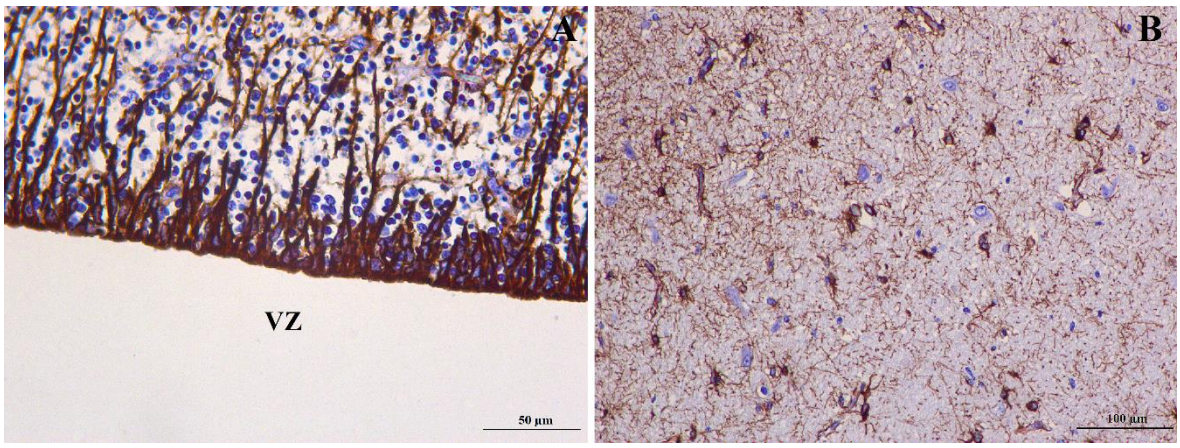

Figure 42. Gestational week 30; immunostaining for GPAP in A) the ventricular zone including ependymal cells and truncated radial glia fibers and in B) in glial cells of intermediate zone

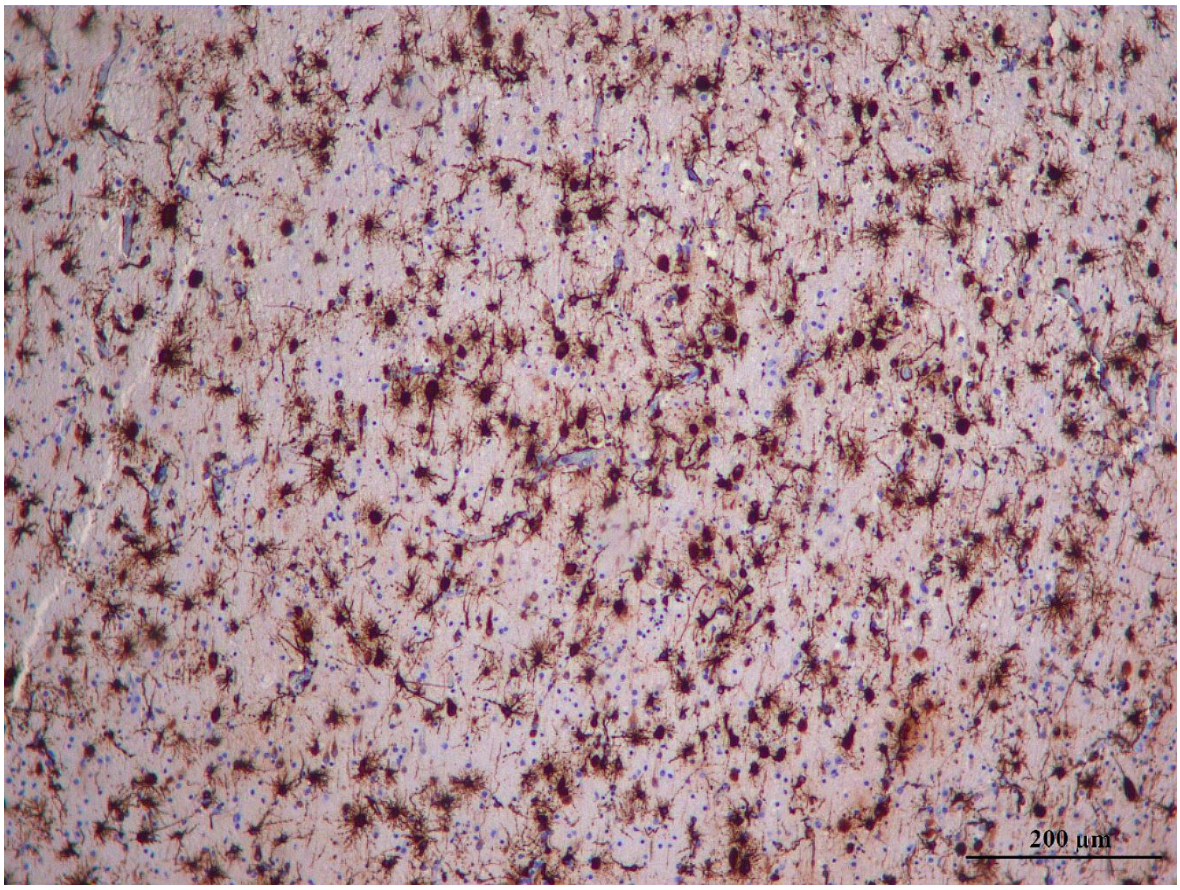

Figure 43. Strong nuclear and cytoplasmic expression of GFAP in mature glial cell at gestational week 39

4.3.2 S100B

From the 9th to the 18th week of gestation, immunoreactivity for S100B was detected in the nuclei and cytoplasm of scattered cells mainly localized in the intermediate zone and cortical plate (Fig. 44). No significant staining was observed in the ventricular zone and in the subventricular zone.

At the 21st week of gestation S100B nuclear and cytoplasmic immunoreactivity was detected in subventricular zone. Moreover, this gestational age was characterized by an increase of S100B reactivity in both cortical plate and intermediate zone (Fig. 45).

At the 24th week, nuclear and cytoplasmic immunostaining was detected in ventricular zone including ependymal cells (Fig. 46A). Moreover, a strong cytoplasmic and nuclear immunoreactivity for S100B was detected in astrocytes in the intermediate zone associated with a weaker immunostaining in the cortical layers (Fig. 46B).

After the 30th week of gestation until the end of gestation S100B nuclear and cytoplasmic immunostaining was particularly strong in astrocytes in both grey and white matter (Fig. 46).

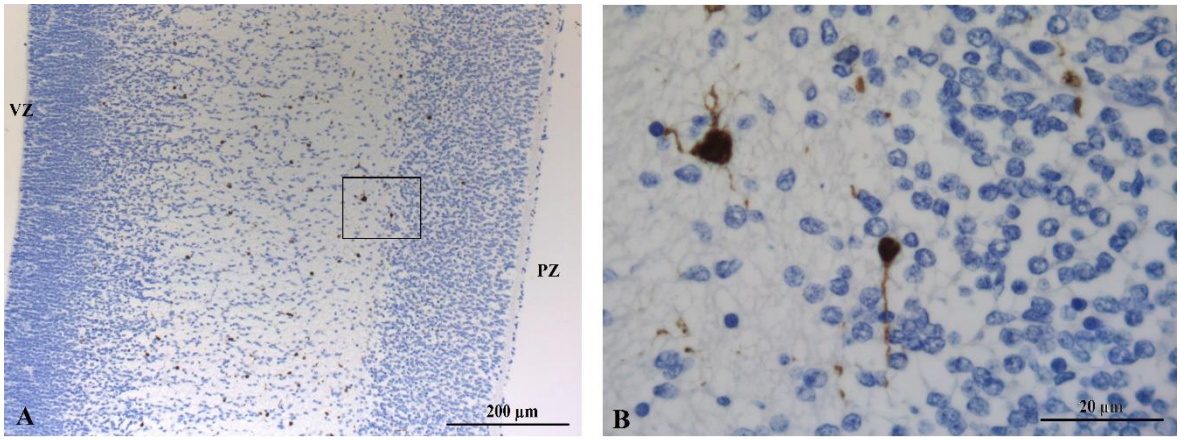

Figure 44. Gestational week 10. A) Nuclear and cytoplasmic immunoreactivity for S100B in scattered cells localized in the intermediate zone; B) Higher magnification of the S100B reactive cells in the intermediate zone

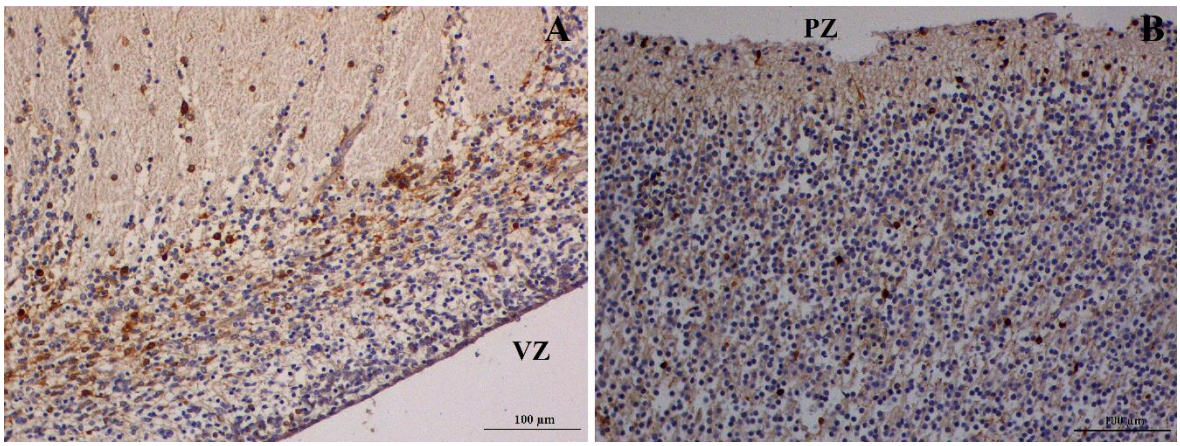

Figure 45. Gestational week 21. S100B nuclear and cytoplasmic immunoreactivity was detected A) in subventricular zone and in B) cortical plate and intermediate zone

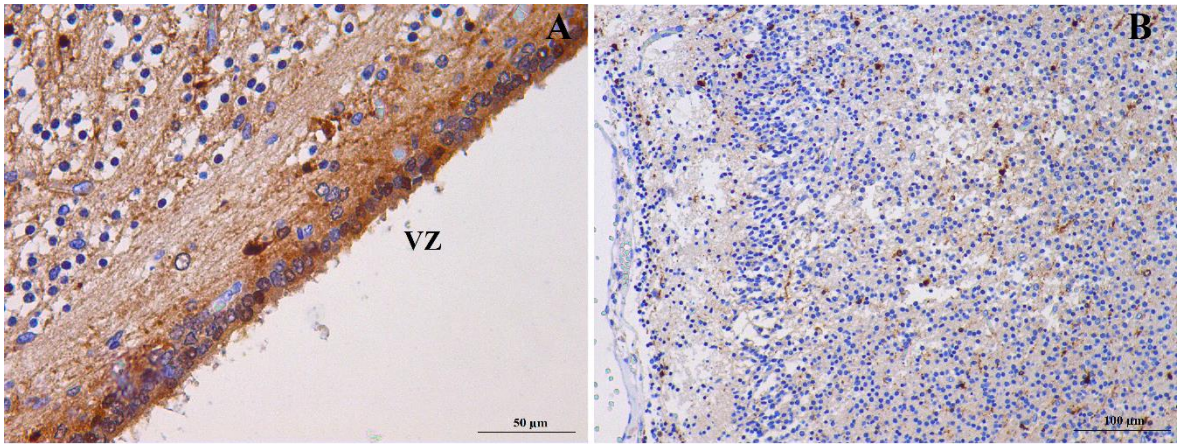

Figure 46. Gestational week 24. A) S100B immunostaining in ventricular zone including ependymal cells in contrast with B) a weaker immunostaining in the cortical layers

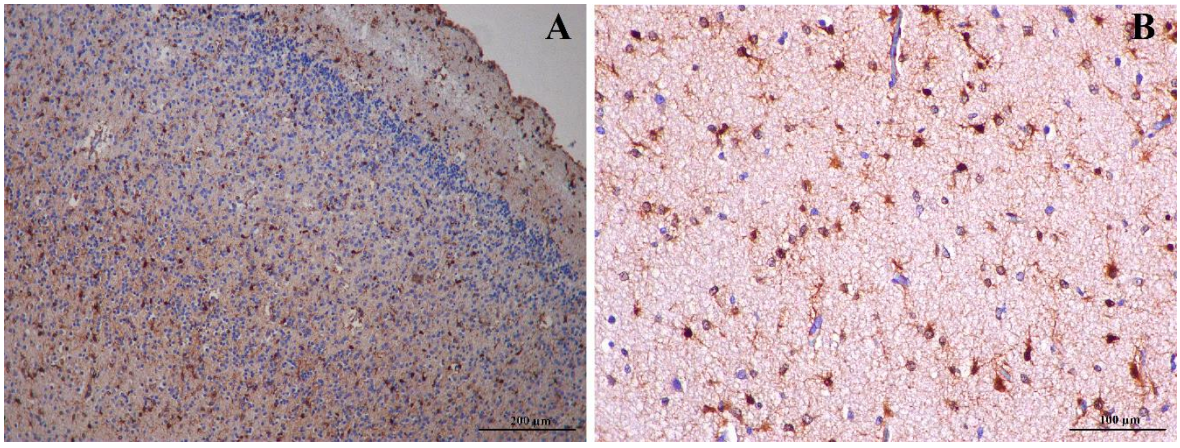

Figure 47. Immunostaining for S100B in astrocytes at A) gestational week 30 and B) gestational week 39

5. Discussion

The development of the human cerebral cortex represents a delicate period of embryogenesis, being characterized by critical molecular and cellular events including proliferation, migration and differentiation of multiple cell types that may not be completely identified by morphology. Previous studies, mainly carried out on experimental models, evidenced that immunohistochemistry may allow the identification of different neural and glial precursors inside the different developing cerebral cortex zones. This study confirms previous reports [83;85] on human corticogenesis carried out by immunohistochemistry as a useful tool for the identification of stem/progenitor cells. Indeed, immunoractivity for several cell markers allows the identification of different stages of differentiation of the neuronal and glial lineages.

As it has already been described, human cortical neurogenesis begins around gestational week 5 in the ventricular zone when NECs undergo asymmetric division giving rise to the main progenitor cells of the developing cerebral cortex, radial glia cells and intermediate progenitor cells, which are generally considered to represent the major source of cortical neurons. Cortical newborn neurons proliferate with a rapid phase from gestational week 10 and a slower phase from gestational week 22 [8].

The ventricular and suventricular zones are considered the “stem/progenitor cell niches” of the developing cerebral cortex being constituted by stem cells, apical and basal progenitors and by the surrounding microenvironment including blood vessels that are important for proper patterning of neurogenesis ensuring its supply with oxygen and nutrients.

The most relevant finding of this study is the ability of the different immunohistochemical markers to immunostain the different zones and cell types of the developing cortex, in particular the stem/progenitor cells niches. Immunohistochemistry

allow identifying the presence of radial glia cells inside the ventricular zone and their projections along the whole cerebral cortex. These findings evidenced five important markers of radial glial cells in both ventricular and subventricular zones: Sox2, Pax6, Vimentin, Nestin and WT1. Vimentin, Nestin and WT1 cytoplasmic expression highlighted the function of radial glia cells that, thanks to their parallel long fibers, represent the most important guide for the radial migration of newborn neurons from the ventricular zone toward the pial zone. In this study, the expression of these markers in radial glia fibers have been detected between the 9th and the 21st gestational week, confirming that once the neuronal migration is complete the radial glia fibers in the subcortical layers disappear. Moreover, since neurogenesis is complete, radial glia cells lose their scaffold role for migrating newborn neurons differentiating in glial lineages giving rise to astrocytes, oligodendrocytes or ependymal cells [116].

Among these three radial glial markers, Vimentin and Nestin appeared the most specific ones, while WT1 was less selective. Nuclear expression of Pax6 and Sox2 allow to evidence the stem/progenitor niches during cortical neurogenesis. Pax6 immunoreactivity was detected from the 9th to the 17th week of gestation in the ventricular and subventricular zones, being expressed in apical and basal radial glia cell bodies. Instead, Sox2 expression was found both in radial glia cells bodies and in intermediate progenitor cells of the two niches. Moreover, immunostaining for Sox2 was detected in newborn neuron migrating from ventricular and subventricular zones toward the cortical plate. The strongest expression has been found between the 9th and the 21st gestational week.

Another interesting finding emerging from this study is the expression of the two transcription factors Pax2 and Sox11. Pax2 nuclear immunoreactivity was detected in all the developing cerebral cortex zones during neurogenic phases. Indeed, Pax2 marks the progenitor cells of ventricular and subventricular zones and the migrating newborn neurons of the intermediate zone, the cortical plate and pial zone. Its expression decreases with the

advancement of gestation showing, after the 34th week, a mild immunoreactivity in scattered cells in the cerebral cortex layers. Sox11 nuclear immunoreactivity was detected mainly in the first half of gestation in the intermediate progenitor cells and newborn neurons of the ventricular, subventricular, intermediate zones and cortical plate. Until the 12th week of gestation the ventricular zone showed several Sox11-reactive cells whereas between the 15th and the 19th week of gestation only scattered cells Sox11-positive were detected intermingled with no-reactive stem/progenitor cells. After the 21st week of gestation, a mild Sox11 nuclear immunostaining was mainly detected in postmitotic cells of the cortical layers. Given their transient expression in certain gestational ages, Sox11 and Pax2 may evidence the differentiation of neural progenitor cells in newborn neurons. Once these neurons reach their destination in the cortical plate, they lose the expression of these markers and begin to express the typical marker of mature neurons.

Moreover, the expression of these markers allow to better understand the different phases of neurogenesis. The *proliferation phase* of stem/progenitor cells and newborn neurons was mainly detected from the 9th to the 12th week of gestation, when the ventricular and subventricular zones had the greatest thickness. The *migration phase* was mainly detected between the 15th to the 18th week of gestation when the ventricular and subventricular zones decrease their thickness and newborn neurons increase the migration toward their final destination in the cortical plate. At these gestational ages, begin the *differentiation phase* of progenitor cells in newborn neuron, which continues during the second half of gestation with the complete neuronal maturation consisting in the growth of neuronal dendrites and axons and the formation of synapses.

The peculiar expression of the different stem/progenitor cells immunohistochemical markers in the developing human cerebral cortex deserves some considerations.

WT1 is a transcription factor highly expressed in several human organs during embryogenesis, including mouse and human nervous system development. In this study, the

strong expression of WT1 in radial glia fibers in the first half of gestation indicates a possible role for this transcription factor in cell migration and differentiation during neurogenesis. These findings added new data on previous studies of our group regarding WT1 expression in radial glia fibers in early stages of CNS development [79], including developing cerebral cortex [83]. WT1 may be considered a useful marker of radial glia cell during human brain development.

Nestin has been reported to be expressed by the primitive neuroephitelium stem cells [85]. In this study, Nestin immunostaining in the first half of gestation was mainly localized in radial glia cells in the ventricular zone and in radial glia projections in the intermediate zone and cortical plate. After the 24th week of gestation, the expression of Nestin decreases until it disappears towards the end of gestation. These findings confirm previous data of our group [83] and consider Nestin as possible important molecular marker involved in neural proliferation and migration during early phases of human cerebral cortex development. Moreover, this study clearly indicates Vimentin as another good marker of radial glia cells during the first half of gestation, suggesting the utility of Vimentin in the detection of early progenitor cells in the developing cerebral cortex of experimental animals [94-96] and in human fetuses [83].

This study showed a prevalent Sox2 nuclear expression in the cells of the ventricular and subventricular zones, suggesting that this transcription factor represent the earliest stem/progenitor cell marker of the developing human cerebral cortex [83] and confirm previous experimental studies on mouse [51] and on human neocortex [35]. Sox2 play an important role during proliferation, migration and differentiation of neural progenitor cells.

The nuclear expression of Pax6 was found in radial glia cells in early phases of cortical neurogenesis confirming previous studies of developing mammalian cerebral cortex [111;35]. Pax6 may play a role in cell proliferation and differentiation of stem/progenitor cells of the developing human cerebral cortex. Moreover, Pax6 and Pax2 interact during

CNS development, particularly during the neural tube and mammalian eye development [100;102]. Findings of this study suggest Pax2 as possible molecular marker involved in cell proliferation, migration and differentiation in mature neurons.

Another interesting finding of this study was the expression of Sox11 in intermediate progenitor cells and in newborn neurons in the first half of human gestation, confirming previous data on experimental animal models [63]. These data suggest that Sox11 may play an important role regulating the proliferation and neuronal differentiation of intermediate progenitor cells during human corticogenesis.

Regarding the expression of the mature neurons and glia markers, the absence of immunoreactivity of NF, GFAP and Synaptophysin between the 9th and the 15th week of gestation reflects the lack of maturation of glial and neuronal cells in the early phases of human corticogenesis.

NSE is a glycolytic isoenzyme expressed in central and peripheral neurons and in neuroendocrine cells [117]. It is generally considered a marker of neural differentiation [118]. Among the specific mature neuron markers, NSE was the first to be expressed by postmitotic cells in intermediate zone, cortical plate and pial zone between the 9th and the 12th week of gestation. Cytoplasmic and nuclear NSE immunoreactivity increases with the progression of the gestation in neural cells in the absence of any reactivity of glial cells.

Synaptophysin, a synaptic vesicle glycoprotein, is present in neuroendocrine cells and in mature neurons of brain and spinal cord in which participate in synaptic transmission regulating the kinetic of synaptic vesicle endocytosis [119]. In this study, mature neurons of developing cerebral cortex begin to express synaptophysyn at the 15th gestational week with a mild immunoreactivity and then progressively increase after the 17th week until the end of gestation.

Neurofilament, an intermediate filament, represents a major component of the neuronal cytoskeleton being particularly abundant in axons. In this study, the expression of

NF in the developing human cerebral cortex has been detected after the 30th gestational week, when postmitotic neurons have developed the typical characteristics of mature neurons, i.e. axons and dendrites. Indeed, Neurofilaments are essential for the radial growth of axons during development, the maintenance of axon caliber and the transmission of electrical impulses along axons [120].

S100B, a protein of the S-100 protein family, represents a glial-specific marker being expressed primarily by astrocytes [121]. Previous studies showed the expression of this protein in normal human fetal hippocampus, entorhinal cortex and occipital cortex [122]. S100B reactivity between the 9th and the 21st week of gestation was restricted to scattered cells in the intermediate zone, whose morphology may be suggestive for their glial lineage. After the 24th gestational week, S100B was express in mature astrocytes of developing cerebral cortex.

Glial fibrillary acidic protein (GFAP) is an intermediate filament protein expressed by glial cells of CNS, including astrocytes and ependymal cells [123]. The expression of GFAP was detected from the 21st week of gestation, in the ventricular zone and in scattered immature glial cells in subventricular and intermediate zones. After 30 weeks of gestation, cytoplasmic GFAP immunostaining was detected in developing and mature glial cells of all cerebral cortex zones, including the developing ependymal cells in the ventricular zone.

These data of these two mature glia markers confirm that gliogenesis mainly occurs in the second half of gestation. Indeed, since the neuronal migration is almost complete, radial glia progenitor cells differentiate in glial lineages.

6. Conclusions

In conclusion, this study shows the relevant role of immunohistochemistry in the detection of multiple stages of differentiation of neuronal and glial progenitors in the developing human cerebral cortex. Future studies are needed to detect differences in the mRNA expression patterns of these markers in the cerebral cortex and to test other stem/progenitor cells markers at different gestational ages, in order to better evaluate their role in cell proliferation and differentiation during human intrauterine development, postnatal and adult life.

References

1. Indredavik MS, Vik T, Evensen KA, Skranes J, Taraldsen G, Brubakk AM. Perinatal risk and psychiatric outcome in adolescents born preterm with very low birth weight or term small for gestational age. J Dev Behav Pediatr. 2010;31(4):286-94.
2. Whitaker AH, Feldman JF, Lorenz JM, McNicholas F, Fisher PW, Shen S, Pinto-Martin J, Shaffer D, Paneth N. Neonatal head ultrasound abnormalities in preterm infants and adolescent psychiatric disorders. Arch Gen Psychiatry. 2011;68(7):742-52.
3. Lindvall O, Björklund A. Cell therapy in Parkinson's disease. NeuroRx. 2004;1(4):382-93.
4. Lindvall O, Kokaia Z, Martinez-Serrano A. Stem cell therapy for human neurodegenerative disorders-how to make it work. Nat Med. 2004;10 Suppl:S42-50.
5. Faa G, Marcialis MA, Ravarino A, Piras M, Pintus MC, Fanos V. Fetal programming of the human brain: is there a link with insurgence of neurodegenerative disorders in adulthood? Curr Med Chem. 2014;21(33):3854-76.
6. Schwarz SC, Schwarz J. Translation of stem cell therapy for neurological diseases. Transl Res. 2010;156(3):155-60.
7. Stiles J, Jernigan TL. The basics of brain development. Neuropsychol Rev. 2010;20(4):327-48.
8. Budday S, Steinmann P, Kuhl E. Physical biology of human brain development. Front Cell Neurosci. 2015;8;9:257.
9. Blows WT. Child brain development. Nurs Times. 2003;99,28–31.
10. Sun T, Hevner RF. Growth and folding of the mammalian cerebral cortex: from molecules to malformations. Nat Rev Neurosci. 2014;15,217–232.
11. Huttenlocher PR, Dabholkar AS. Regional differences in synaptogenesis in human cerebral cortex. J Compar Neurol. 1997;387,167–178.

12. Richman DP, Stewart RM, Hutchinson JW, Caviness VS Jr. Mechanical model of brain convolutional development. Science. 1975;189(4196):18-21.
13. Craik FIM, Bialystok E.Cognition through the lifespan: mechanisms of change. Trends Cogn Sci. 2006;10,131–138.
14. Kandel, ER, Schwartz JH, Jessel TM. Principles of Neural Science. McGraw-Hill Professional. 2000; p. 324. ISBN 978-0-8385-7701-1.
15. Pakkenberg B, Pelvig D, Marner L, Bundgaard MJ, Gundersen HJ, Nyengaard JR, Regeur L. Aging and the human neocortex. Exp Gerontol. 2003;38(1-2):95-9.
16. Ransohoff RM, Cardona AE. The myeloid cells of the central nervous system parenchyma. Nature. 2010;468(7321):253-62.
17. Ginhoux F, Greter M, Leboeuf M, Nandi S, See P, Gokhan S, Mehler MF, Conway SJ, Ng LG, Stanley ER, Samokhvalov IM, Merad M. Fate mapping analysis reveals that adult microglia derive from primitive macrophages. Science. 2010;330:841-845.
18. Greter M, Merad M. Regulation of microglia development and homeostasis. Glia. 2013;61:121-127
19. Anderson DJ, Gage FH, Weissman IL. Can stem cells cross lineage boundaries? Nat Med. 2001;7:393-395.
20. De Felici M, Boitani C, Bouchè M, Canipari R, Dolfi A. Embriologia umana. Morfogenesi. Processi Molecolari. Aspetti clinici. Piccin Editore 2013
21. Hima Bindu A, Srilatha B. Potency of Various Types of Stem Cells and their Transplantation. J Stem Cell Res Ther. 2011;1:115.
22. Gremo F, Presta M. Role of fibroblast growth factor-2 in human brain: a focus on development. International Journal of Developmental Neuroscience. Volume 18, Issues 2–3, June 2000, Pages 271–279

23. Garcez RC, Teixeira BL, Schmitt Sdos S, Alvarez-Silva M, Trentin AG. Epidermal growth factor (EGF) promotes the in vitro differentiation of neural crest cells to neurons and melanocytes. Cell Mol Neurobiol. 2009;29(8):1087-91.
24. Ho KS, Scott MP. Sonic hedgehog in the nervous system: functions, modifications and mechanisms. Curr Opin Neurobiol. 2002;12(1):57-63.
25. Kalani MY, Cheshier SH, Cord BJ, Bababeygy SR, Vogel H, Weissman IL et al. Wnt-mediated self-renewal of neural stem/progenitor cells. Proc Natl Acad Sci U S A. 2008;105(44):16970-5.
26. Götz M, Huttner WB. The cell biology of neurogenesis. Nat Rev Mol Cell Biol. 2005;6(10):777-88.
27. Florio M, Huttner WB. Neural progenitors, neurogenesis and the evolution of the neocortex. Development. 2014;141(11):2182-94.
28. Malatesta P, Appolloni I, Calzolari F. Radial glia and neural stem cells. Cell Tissue Res. 2008;331(1):165-78.
29. Rakic S, Zecevic N. Emerging complexity of layer in human cerebral cortex. Cereb. Cortex. 2003;13,1072–1083.
30. Raybaud C, Ahmad T, Rastegar N, Shroff M and Nassar A. M. The premature brain: developmental and lesional anatomy. Neuroradiology. 2013;55,23–40.
31. Molnár Z, Adams R, Blakemore C. Mechanisms underlying the early establishment of thalamo cortical connections in the rat. J Neurosci. 1998;18,5723–5745.
32. Zecevic N, Chen Y, Filipovic R. Contributions of cortical sub-ventricular zone to the development of the human cerebral cortex. J Comp Neurol 2005;491:109–122.
33. Hansen DV, Lui JH, Parker PR, Kriegstein AR. Neurogenic radial glia in the outer subventricular zone of human neocortex. Nature. 2010 Mar 25;464(7288):554-561.
34. Campbell K, Götz M. Radial glia: multi-purpose cells for vertebrate brain development. Trends Neurosci. 2002;25(5):235-8.

35. Pollen AA, Nowakowski TJ, Chen J, Retallack H, Sandoval-Espinosa C, Nicholas CR, et al. Molecular identity of human outer radial glia during cortical development. Cell. 2015;163(1):55-67.
36. Lui JH, Hansen DV , Kriegstein AR. Development and evolution of the human neocortex. Cell. 2011;146,18–36.
37. Bystron I ,Blakemore C, Rakic P. Development of the human cerebral cortex: boulder committee revisited. Nat. Rev. Neurosci. 2008;9,110–122.
38. Smart I, Lablond CP. Evidence for division and transformations of neuroglia cells in the mouse brain, as derived from radioautography after injection of thymidine-H3. Journal of Comparative Neurology Volume 116, Issue 3, pages 349–367, June 1961.
39. Altman J, Das GD. Post-natal origin of microneurones in the rat brain. Nature 1965;207:953–956.
40. Altman J, Das GD. Autoradiographic and histological studies of postnatal neurogenesis. I. A longitudinal investigation of the kinetics, migration and transformation of cells incorporating tritiated thymidine in neonate rats, with special reference to postnatal neurogenesis in some brain regions. J Comp Neurol. 1966;126(3):337-89.
41. Altman J. Autoradiographic and histological studies of postnatal neurogenesis. IV. Cell proliferation and migration in the anterior forebrain, with special reference to persisting neurogenesis in the olfactory bulb. J Comp Neurol. 1969;137(4):433-57.
42. Eriksson PS, Perfilieva E, Björk-Eriksson T, Alborn AM, Nordborg C, Peterson DA, Gage FH. Neurogenesis in the adult human hippocampus. Nat Med. 1998;4(11):1313-7.
43. Braun SM, Jessberger S. Adult neurogenesis: mechanisms and functional significance. Development. 2014;141(10):1983-6.

44. Lepousez G, Valley MT Lledo PM. The impact of adult neurogenesis on olfactory bulb circuits and computations. Annu Rev Physiol. 2013;75, 339-363.
45. Deng W, Aimone JB, Gage FH. New neurons and new memories: how does adult hippocampal neurogenesis affect learning and memory? Nat Rev Neurosci. 2010;11(5):339-50.
46. Neves G, Cooke SF, Bliss TV. Synaptic plasticity, memory and the hippocampus: a neural network approach to causality. Nat Rev Neurosci. 2008;9(1):65-75.
47. Santarelli L, Saxe M, Gross C, Surget A, Battaglia F, Dulawa S, Weisstaub N, Lee J, Duman R, Arancio O, Belzung C, Hen R. Requirement of hippocampal neurogenesis for the behavioral effects of antidepressants. Science. 2003;301(5634):805-9.
48. Zhao CS, Overstreet-Wadiche L. Integration of adult generated neurons during epileptogenesis. Epilepsia. 2008;49 Suppl 5:3-12.
49. Donovan MH, Yazdani U, Norris RD, Games D, German DC, Eisch AJ. Decreased adult hippocampal neurogenesis in the PDAPP mouse model of Alzheimer's disease. J Comp Neurol. 2006;1;495(1):70-83.
50. Gubbay J, Collignon J, Koopman P, Capel B, Economou A, Munsterberg A, Vivian N, Goodfellow P, Lovell-Badge R. A gene mapping to the sex-determining region of the mouse Y chromosome is a member of a novel family of embryonically expressed genes. Nature. 1990;346:245–250
51. Bani-Yaghoub M, Tremblay RG, Lei JX, Zhang D, Zurakowski B, Sandhu K, et al. Role of Sox2 in the development of the mouse neocortex. Dev Biol. 2006;295:52-66.
52. Sarkar A and Hochedlinger K. The SOX family of transcription factors: versatile regulators of stem and progenitor cell fate. Cell Stem Cell 2013;12:15-30.

53. Wegner M, Stolt CC. From stem cells to neurons and glia: a Soxist's view of neural development. Trends Neurosci. 2005; 28: 583-588.

54. Thiel G. How Sox2 maintains neural stem cell identity. Biochem J. 2013;450(3):e1-2.

55. Graham V, Khudyakov J, Ellis P, Pevny L. SOX2 functions to maintain neural progenitor identity. Neuron. 2003; 39: 749-765

56. Fong H, Hohenstein KA, Donovan PJ. Regulation of self-renewal and pluripotency by Sox2 in human embryonic stem cells. Stem Cells 2008; 26: 1931-1938

57. Zhang S, Cui W. Sox2, a key factor in the regulation of pluripotency and neural differentiation. World J Stem Cells. 2014; 6(3): 305–311.

58. Wang Z, Oron E, Nelson B, Razis S, Ivanova N. Distinct lineage specification roles for NANOG, OCT4, and SOX2 in human embryonic stem cells. Cell Stem Cell 2012;10: 440-454

59. Bergsland M, Werme M, Malewicz M, Perlmann T and Muhr J. The establishment of neuronal properties is controlled by Sox4 and Sox11. Genes Dev. 2006;20: 3475–3486.

60. Haslinger A, Schwarz TJ, Covic M and Chichung Lie D. Expression of Sox11 in adult neurogenic niches suggests a stage-specific role in adult neurogenesis. Eur J Neurosci 2009, 29: 2103–2114.

61. Mu L, Berti L, Masserdotti G, Covic M, Michaelidis TM, Doberauer K and Merz K, et al. SoxC transcription factors are required for neuronal differentiation in adult hippocampal neurogenesis. J Neurosci. 2012, 32:3067–3080.

62. Lin L, Lee VM, Wang Y, Lin JS, Sock E, Wegner M, Lei L. Sox11 regulates survival and axonal growth of embryonic sensory neurons. Dev Dyn. 2011;240, 52–64.

63. Li Y, Wang J, Zheng Y, Zhao Y, Guo M, Li Y, Bao Q, Zhang Y, Yang L, Li Q. Sox11 modulates neocortical development by regulating the proliferation and

neuronal differentiation of cortical intermediate precursors. Acta Biochim Biophys Sin (Shanghai). 2012;44(8):660-8.

64. Chen C, Lee GA, Pourmorady A, Sock E, Donoghue MJ. Orchestration of neuronal differentiation and progenitor pool expansion in the developing cortex by SoxC genes. J Neurosci. 2015;35, 10629-10642.
65. Chen C, Jin J, Lee GA, Silva E, Donoghue M. Cross-species functional analyses reveal shared and separate roles for Sox11 in frog primary neurogenesis and mouse cortical neuronal differentiation. Biology Open. 2016; doi: 10.1242/bio.015404
66. Hoshiba Y, Toda T, Ebisu H, Wakimoto M, Yanagi S, Kawasaki H. Sox11 Balances Dendritic Morphogenesis with Neuronal Migration in the Developing Cerebral Cortex. J Neurosci. 2016;36(21):5775-84.
67. Hastie ND. The genetics of Wilms' tumor - a case of disrupted development. Annu Rev Genet. 1994;523–558.
68. Call KM, Glaser T, Ito CY, Buckler AJ, Pelletier J, Haber DA, et al. Isolation and characterization of a zinc finger polypeptide gene at the human chromosome 11 Wilms' tumor locus. Cell. 1990;60: 509–520.
69. Rauscher FJ 3rd, Morris JF, Tournay OE, Cook DM, Curran T. Binding of the Wilms' tumor locus zinc finger protein to the EGR-1 consensus sequence. Science. 1990;250(4985):1259-62.
70. Hamilton TB, Barilla KC, Romaniuk PJ. High affinity binding sites for the Wilms' tumour suppressor protein WT1. Nucleic Acids Res. 1995;23(2):277-84.
71. Haber DA, Sohn RL, Buckler AJ, Pelletier J, Call KM, Housman DE. Alternative splicing and genomic structure of the Wilms tumor gene WT1. Proc Natl Acad Sci USA. 1991;88: 9618–9622
72. Sharma PM, Bowman M, Madden SL, Rauscher FJ 3rd, Sukumar S. RNA editing in the Wilms' tumor susceptibility gene, WT1. Genes Dev. 1994;8: 720–731.

73. Hammes A, Guo JK, Lutsch G, Leheste JR, Landrock D, Ziegler U, et al. Two splice variants of the Wilms' tumor 1 gene have distinct functions during sex determination and nephron formation. Cell. 2001;106: 319–329.
74. Lee SB, Haber DA. Wilms tumor and the WT1 gene. Exp Cell Res. 2001; 264(1):74-99.
75. Moore AW, McInnes L, Kreidberg J, Hastie ND, Schedl A. YAC complementation shows a requirement for Wt1 in the development of epicardium, adrenal gland and throughout nephrogenesis. Development. 1999;126(9):1845-57.
76. Herzer U, Crocoll A, Barton D, Howells N, Englert C. The Wilms tumor suppressor gene wt1 is required for development of the spleen. Curr Biol. 1999;9(15):837-40.
77. Wagner KD, Wagner N, Vidal VP, Schley G, Wilhelm D, Schedl A, et al. The Wilms' tumor gene Wt1 is required for normal development of the retina. EMBO J. 2002;21(6):1398-405.
78. Wagner N, Wagner KD, Hammes A, Kirschner KM, Vidal VP, Schedl A, et al. A splice variant of the Wilms' tumour suppressor WT1 is required for normal development of the olfactory system. Development. 2005;132:1327–36.
79. Ambu R, Vinci L, Gerosa C, Fanni D, Obinu E, Faa A, Fanos V. WT1 expression in the human fetus during development. Eur J Histochem. 2015;59(2):2499.
80. Parenti R, Salvatorelli L, Musumeci G, Parenti C, Giorlandino A, Motta F, Magro G. Wilms' tumor 1 (WT1) protein expression in human developing tissues. Acta Histochem. 2015;117(4-5):386-96.
81. Fanni D, Fanos V, Monga G, Gerosa C, Locci A, Nemolato S, et al. Expression of WT1 during normal human kidney development. J Matern Fetal Neonatal Med. 2011;24 Suppl 2:44-7.

82. Sanna A, Fanos V, Gerosa C, Vinci L, Puddu M, Loddo C, Faa G. Immunohistochemical markers of stem/progenitor cells in the developing human kidney. Acta Histochem. 2015 May-Jun;117(4-5):437-43.
83. Vinci L, Ravarino A, Fanos V, Naccarato AG, Senes G, Gerosa C, Bevilacqua G, Faa G, Ambu R. Immunohistochemical markers of neural progenitor cells in the early embryonic human cerebral cortex. Eur J Histochem. 2016;60(1):2563. doi: 10.4081/ejh.2016.2563
84. Liu J, Ji X, Li Z, Zheng H, Zheng W, Jia J, Shen H, Zhang Q, An J. Nestin overexpression promotes the embryonic development of heart and brain through the regulation of cell proliferation. Brain Res. 2015;1610:1-11.
85. Lendahl U, Zimmerman LB, McKay RD. CNS stem cells express a new class of intermediate filament protein. Cell. 1990;60(4):585-95.
86. Gilyarov AV. Nestin in central nervous system cells. Neurosci Behav Physiol. 2008;38(2):165-9.
87. Yan S, Li P, Wang Y, Yu W, Qin A, Liu M, Xiang AP, Zhang W, Li W. Nestin regulates neural stem cell migration via controlling the cell contractility. Int J Biochem Cell Biol. 2016;78:349-60
88. Halliday GM, Cullen KM, Kril JJ, Harding AJ, Harasty J. Glial fibrillary acidic protein (GFAP) immunohistochemistry in human cortex: a quantitative study using different antisera. Neurosci Lett. 1996;209(1):29-32.
89. Melissa G. Mendez, Shin-Ichiro Kojima, and Robert D. Goldman. Vimentin induces changes in cell shape, motility, and adhesion during the epithelial to mesenchymal transition. FASEB J. 2010; 24(6): 1838–1851.
90. Mendez MG, Kojima S, Goldman RD. Vimentin induces changes in cell shape, motility, and adhesion during the epithelial to mesenchymal transition. FASEB J. 2010;24(6):1838-51.

91. Satelli A, Li S. Vimentin in cancer and its potential as a molecular target for cancer therapy. Cell Mol Life Sci. 2011;68(18):3033-46.
92. Martha E. Kidd, Dale K. Shumaker, and Karen M. Ridge. The Role of Vimentin Intermediate Filaments in the Progression of Lung Cancer. Am J Respir Cell Mol Biol. 2014; 50(1): 1–6.
93. Kálmán M, Ajtai BM. A comparison of intermediate filament markers for presumptive astroglia in the developing rat neocortex: immunostaining against nestin reveals more detail, than GFAP or vimentin. Int J Dev Neurosci. 2001;19(1):101-8.
94. Oudega M, Marani E. Expression of vimentin and glial fibrillary acidic protein in the developing rat spinal cord: an immunocytochemical study of the spinal cord glial system. J Anat. 1991;179:97-114.
95. Zecevic N, Mlosevic A, Rakic S, Marin-Padilla M. The human neocortex: early development and composition of the primordial plexiform layer. An immunohistochemical study. J Comp Neurol 1999;412:241–254.
96. Zecevic N. Specific characteristic of radial glia in the human fetal telencephalon. Glia. 2004;48(1):27-35.
97. Wehr R, Gruss P. Pax and vertebrate development. Int J Dev Biol. 1996. 40: 369–377.
98. Dressler GR. and Douglass EC. Pax-2 is a DNA-binding protein expressed in embryonic kidney and Wilms' tumor. Proc Natl Acad Sci. 1992. USA 89, 1179-1183.
99. Ryan G, Steele-Perkins V, Morris JF, Rauscher FJ 3rd, Dressler GR. Repression of Pax-2 by WT1 during normal kidney development. Development. 1995;121(3):867-75.
100. Schwarz M, Alvarez-Bolado G, Dressler G, Urbanek P, Busslinger M, Gruss, P. Pax 2/5 and Pax6 subdivide the early neural tube into three domains. Mech Dev. 1999;82, 29-39.

101. Matsunaga E, Araki I, Nakamura H. Pax6 defines the di-mesencephalic boundary by repressing En1 and Pax2. Development. 2000;127, 2357-2365.

102. Schwarz M, Cecconi F, Bernier G, Andrejewski N, Kammandel B, Wagner M, Gruss P. Spatial specification of mammalian eye territories by reciprocal transcriptional repression of Pax2 and Pax6. Development. 2000;127, 4325-4334.

103. Terzić J, Muller C, Gajović S, Saraga-Babić M. Expression of PAX2 gene during human development. Int J Dev Biol. 1998;42(5):701-7.

104. Namm A, Arend A, Aunapuu M. Expression of Pax2 protein during the formation of the central nervous system in human embryos. Folia Morphol (Warsz). 2014;73(3):272-8.

105. Glaser T, Walton DS, Maas RL. Genomic structure, evolutionary conservation and aniridia mutations in the human PAX6 gene. Nat Genet. 1992;2,232–239.

106. Jun S, Desplan C. Cooperative interactions between paired domain and homeodomain. Development. 1996;122, 2639–2650.

107. Hill RE, Favor J, Hogan BL, Ton CC, Saunders GF, Hanson IM, Prosser J, Jordan T, Hastie ND, vanHeyningen V. Mouse small eye results from mutations in a paired-like homeobox-containing gene. Nature. 1991;354,522–525.

108. Hever AM, Williamson KA, van Heyningen V. Developmental malformations of the eye: the role of PAX6, SOX2 and OTX2. Clin Genet. 2006;69(6):459-70.

109. Georgala PA, Carr C B, Price DJ. (2011a).The role of Pax6 in forebrain development. Dev. Neurobiol. 71, 690–709.

110. Manuel MN, Mi D, Mason JO, Price DJ. Regulation of cerebral cortical neurogenesis by the Pax6 transcription factor. Front Cell Neurosci. 2015;9:70.

111. Gotz M, Stoykova A, Gruss P. Pax6 controls radial glia differentiation in the cerebral cortex. Neuron. 1998;21, 1031–1044.

112. Warren N, Caric D, Pratt T, Clausen JA, Asavaritikrai P, Mason JO et al. The transcription factor, Pax6, is required for cell proliferation and differentiation in the developing cerebral cortex. Cereb Cortex.1999;9, 627–635.

113. Sessa A, Mao CA, Colasante G, Nini A, Klein WH, Broccoli V. Tbr2-positive intermediate (basal) neuronal progenitors safeguard cerebral cortex expansion by controlling amplification of pallial glutamatergic neurons and attraction of subpallial GABAergic interneurons. Genes Dev. 2010;24(16):1816-26.

114. Englund C, Fink A, Lau C, Pham D, Daza RA, Bulfone A, Kowalczyk T, Hevner RF. Pax6, Tbr2, and Tbr1 are expressed sequentially by radial glia, intermediate progenitor cells, and postmitotic neurons in developing neocortex. J Neurosci. 2005;25(1):247-51.

115. Vinci L, Ravarino A, Gerosa C, Pintus MC, Marcialis MA, Marinelli V, Faa G, Fanos V, Ambu R. Stem/progenitor cells in the cerebral cortex of the human preterm: a resource for an endogenous regenerative neuronal medicine? J Pediatr Neonat Individual Med. 2016;5(1):e050121.

116. Misson JP, Takahashi T, Caviness VSJ. Ontogeny of radial and other astroglial cells in murine cerebral cortex. Glia. 1991;4:138–148.

117. Marangos PJ, Schmechel DE, Parma AM, Goodwin FK. Developmental profile of neuron-specific (NSE) and non-neuronal (NNE) enolase. Brain Res. 1980;190(1):185-93.

118. Mareschi K, Novara M, Rustichelli D, Ferrero I, Guido D, Carbone E et al. Neural differentiation of human mesenchymal stem cells: Evidence for expression of neural markers and eag K+ channel types. Exp Hematol. 2006;34(11):1563-72.

119. Kwon SE, Chapman ER. Synaptophysin regulates the kinetics of synaptic vesicle endocytosis in central neurons. Neuron. 2011;70(5):847-54.

120. Yuan A, Rao MV, Veeranna, Nixon RA. Neurofilaments at a glance. J Cell Sci. 2012;125: 3257-3263

121. Zimmer DB, Cornwall EH, Landar A, Song W. The S100 protein family: history, function, and expression. Brain Res Bull. 1995;37(4):417-29.

122. Adami C, Sorci G, Blasi E, Agneletti AL, Bistoni F, Donato R. S100B expression in and effects on microglia. Glia. 2001;33(2):131-42.

123. Roessmann U, Velasco ME, Sindely SD, Gambetti P. Glial fibrillary acidic protein (GFAP) in ependymal cells during development. An immunocytochemical study. Brain Res. 1980;200(1):13-21.