Expression of stem cell markers in primary cell cultures and fresh-fixed tissue preparations deriving from nervous tissue of postnatal opossums

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SVEUČILIŠTE U RIJECI

ODJEL ZA BIOTEHNOLOGIJU

Diplomski sveučilišni studij

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Ekspresija markera matičnih stanica u primarnim staničnim kulturama te tkivnim prerezima živčanog tkiva mladih oposuma

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in front of the committee:

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Abstract

Unlike the adult mammalian central nervous system (CNS) that has limited capacity for regeneration, the immature mammalian CNS shows a unique ability to successfully regenerate after injury. Although regeneration studies have provided significant insight into the basic molecular mechanisms underlying CNS regeneration, the key differences between the organisms that can and cannot regenerate are still unknown. *Monodelphis domestica,* commonly known as the gray short-tailed opossum, is a unique preparation that offers an exceptional opportunity to study mammalian CNS regeneration, without a need for intrauterine surgery. Opossums are marsupials born at highly immature developmental stage, with the possibility to regenerate spinal cord after injury in the first two weeks of their lives. The aim of this thesis was to prepare and characterize two different preparations: CNS primary cultures and fresh-fixed spinal cord tissue from the opossums of different age, which can and cannot regenerate after injury. Characterization was performed using immunofluorescence to reveal differences in the cellular content and expression of markers related to regeneration with a focus on stem cells. Also, we tested two different plating mediums to examine the impact of the medium composition on cell growth and differentiation. Firstly, we confirmed that the expression of TUJ1, a neuron-specific marker and the stem/glial progenitor SOX9 is mutually exclusive. Secondly, we confirmed that SOX2 is a powerful tool for characterizing stem/progenitor cells through the CNS. Thirdly, we showed that the medium composition indeed affects cell growth and differentiation. Finally, in spinal cord intact tissue, we confirmed the localization of cells that co-express SOX2 and SOX9. In younger tissue, these cells are present along the central canal, while in older animal cells are scattered. The results of this study will help to develop the tools to study and characterize CNS stem cells that could be used in novel strategies to cure spinal cord injury.

Key words CNS regeneration, opossum, primary cell cultures, immunofluorescence, stem cell markers

Sažetak

Za razliku od središnjeg živčanog sustava (SŽS) odraslog sisavca koji ima ograničeni kapacitet regeneracije, embrionalni SŽS sisavaca iskazuje jedinstvenu sposobnost uspješne regeneracije nakon ozljede. Iako su brojna istraživanja pružila značajan uvid u osnovne molekularne mehanizme koji su temelj regeneracije SŽSa, ključne razlike između organizama koji imaju te onih koji nemaju sposobnost regeneracije, još uvijek su neodređene. *Monodelphis domestica*, poznatiji kao kratkorepi sivi oposum, jedinstveni je model koji pruža izuzetnu priliku za proučavanje regeneracije SŽS sisavaca, bez potrebe za invazivnim operacijama. Oposumi su tobolčari koji se rađaju vrlo nerazvijeni, s mogućnošću potpune regeneracije leđne moždine tijekom prva dva tjedna života. Cilj ovog rada je bio pripremiti i karakterizirati dva različita preparata: primarne stanične kulture SŽSa i tkivne prereze leđne moždine oposuma različite starosti te različite sposobnosti regeneracije. Karakterizacija je provedena uporabom imunofluorescencije s ciljem otkrivanja razlika u staničnom sadržaju te ekspresiji biljega koji su povezani s regeneracijom, posebice biljega matičnih stanica. Također, testirali smo dva različita medija za nasađivanje kako bi ispitali utjecaj sastava medija na rast i diferencijaciju stanica. Potvrdili smo da se ekspresija TUJ1, specifičnog biljega neurona, te biljega matičnih/glijalnih prekursora SOX9 međusobno isključuje. Biljeg SOX2 istaknuo se kao snažan alat za karakterizaciju potencijalnih matičnih stanica u SŽSu. Testiranjem različitih medija pokazali smo da sastav medija doista utječe na rast i diferencijaciju stanica. Konačno, u tkivu leđne moždine oposuma različite dobi posebno se istaknula različita lokalizacija stanica koje eksprimiraju i SOX2 i SOX9. U tkivima mlađih oposuma prisutne su duž središnjeg kanala, dok su u tkivu starijih razasute naokolo. Ovi rezultati doprinjeti će razvitku alata za proučavanje i karakterizaciju matičnih stanica SŽSa, koje se mogu koristiti u strategijama liječenja ozljeda leđne moždine.

Ključne riječi regeneracija SŽSa, oposum, primarne kulture, imunofluorescencija, biljezi matičnih stanica

List of abbreviations

ALS – Amyotrophic lateral sclerosis

BLAST – Basic Local Alignment Search Tool

BME - Basal medium Eagle

BP – Basal progenitor

BSA – Bovine serum albumin

BSB – Blood-spinal cord barrier

cAMP – Cyclic adenosine monophosphate

CC – Central canal

CNS – Central nervous system

CSPG – Chondroitin sulfate proteoglycan

DAPI - 4’,6-diamidino-2-phenylindole

DC – Dorsal column

DIV – days *in vitro*

DMEM - Dulbecco’s modified Eagle medium

E – Embryonic day

ESC – Embryonic stem cell

FBS - Fetal bovine serum

FCS – Fetal calf serum

GM – Glial medium

HBSS - Hanks’ Balanced Salt Solution

HCL – Hydrochloric acid

HEPES - Hydroxyethyl-piperazine-ethane-sulfonic acid

HGM – High-mobility group

ICC - Immunocytochemistry

IF - Immunofluorescence

IHC - Immunohistochemistry

IPC – Intermediate progenitor cell

iPSC – Induced pluripotent stem cell

MAG - Myelin associated glycoprotein

NE – Neuronal medium

NEC – Neuroepithelial cell

NGS - Normal goat serum

NPC – Neural progenitor cell

NSC – Neural stem cell

NT-3 – Neurotrophin - 3

OCT - Optimal cutting temperature

P – Postnatal day

PBS - Phosphate buffer saline

PFA - Paraformaldehyde

RGC – Radial glial cell

RT – Room temperature

SC – Spinal cord

SCI – Spinal cord injury

SD - Standard deviation

SGZ – Subgranular zone

SVZ – Subventricular zone

TBI – Traumatic brain injury

VC – Ventral column

VZ – Ventricular zone

Contents

1. Introduction

1.1 Central nervous system regeneration

The regeneration of the central nervous system (CNS) has always fascinated scientists. The CNS consists of the brain and spinal cord. While the brain is in charge of coordinating higher cognitive and regulatory functions, the spinal cord transfers nerve impulses from the brain to the periphery, and *vice versa*. Traumatic brain injury (TBI) and spinal cord injury (SCI) are the final outcomes of physical or mechanical trauma on the brain or spinal cord tissue (1).

Unlike lower vertebrates, such as fish (2), reptiles, amphibians and adult mammalians have extremely limited capacity to regenerate their CNS after traumatic injury. Since the time of Ramon y Cajal (3), it has been known that damaged CNS neurons are able to sprout after injury, but they are unsuccessful at crossing the lesion or re-creating any connections. In contrast, peripheral nerves can successfully regrow and recover their functionality to a substantial degree after injury.

During the last quarter of the 20th century, a lot of effort was put into studying the regeneration of injured spinal cord in adult as well as embryonic and neonatal animals including hamsters (4), chicks (5), rats (6) and opossums (7). These studies have shown that the capacity for CNS regeneration is lost with development and age progression, meaning that mammalians can successfully repair their brain and spinal cord only at immature developmental stages. Due to the extremely complex interactions involved in regeneration, it is improbable that a single gene is accountable for this failure in regeneration ability. The question that remains unanswered is: What are the differences between organisms that can and cannot regenerate?

Different studies have correlated the loss of regenerative capacity with the development of glial cells. Differentiation of oligodendrocytes and astrocytes represents a dramatic change in the nervous system's cellular composition. The appearance of these cells follows a change in gene expression and growth-inhibitory molecules associated with myelin become up-regulated while growth-promoting molecules become downregulated. Some of the growth-promoting proteins are neurotrophin-3 (NT-3) (8), transcription factor c-Jun (9), and cyclic AMP (cAMP) (10). Growth-inhibitory proteins include myelin-associated protein Nogo-A (formerly neurite inhibitor 250, NI250) (11), myelin-associated glycoprotein (MAG) (12) and chondroitin sulfate proteoglycans (CSPGs) (13) among others.

Moreover, CNS injury triggers glial activation, which ultimately leads to the formation of a glial scar. Glial activation and scar serve as defense mechanisms to SCI and have certain beneficial effects. Glial activation minimizes secondary tissue damage by secluding the lesion area from healthy tissue, removing the tissue debris from the lesion, restoring of the blood–spinal cord barrier (BSB) as well as tissue structure, and maintaining of homeostasis. Even though glial activation shows a neuroprotective role at the initial stage of SCI, gradually it has an inhibitory influence on axonal regeneration (14). This process consists of the recruitment of astrocytes, microglia and oligodendrocyte precursors creating an environment in which regeneration is unsuccessful. As mentioned, most of these cells produce growth-inhibitory molecules. Therefore, glial scar represents a physical as well as chemical barrier to axon regeneration (15). Other, poorly defined, factors are also likely to be involved in failure of axon regeneration.

Intensive research to understand the basic molecular mechanisms underlying CNS regeneration is in progress. By combining different experimental approaches, we are able to improve our knowledge about the loss of regenerative capacity, which will hopefully lead to a successful regeneration strategy. In the last two decades of research, scientists have gained certain insight into intrinsic mechanisms as well as environmental

impact that are involved in failure in CNS regeneration ability after an injury. Although the cure is out of sight, for now, each day we are a step closer than before.

A single therapeutic approach will probably not be enough for the functional repair of severe CNS injury. Upcoming therapeutic approaches focus mainly on neuroprotection (16), promoting the neurite growth (8), preventing the growth-inhibitory molecules (11) and minimizing astroglial scar formation that represents both chemical and physical barrier to neurite regeneration (15).

1.2 The opossum as a preparation for regeneration studies

Monodelphis domestica, generally referred to as the gray short-tailed opossum, is a small rodent-like marsupial from Southern and Eastern Brazil (Fig. 1, A). Opossums are very interesting because, unlike other marsupial species, they do not have a pouch and neonates instead just cling to the teats on the mother's belly (Fig. 1, B).

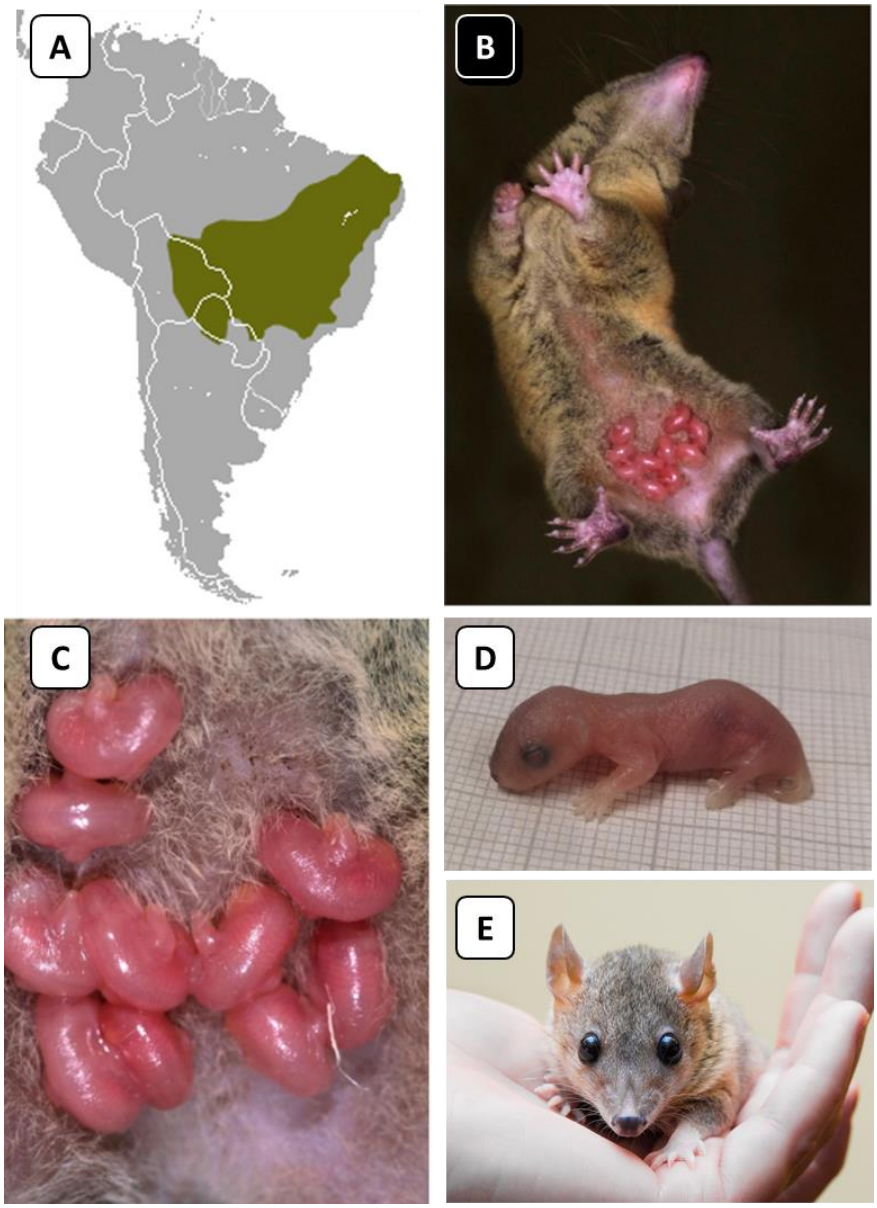


Figure 1. Natural habitat and developmental stages of *Monodelphis domestica*. **A** *Monodelphis domestica* is generally found south of the Amazon River, in southern, eastern, and western Brazil. **B** Female adult with 10 opossum pups attached to the teats. Right after the birth neonates migrate toward the abdomen and remain attached to the teats for a period of up to 2 weeks. **C** Neonatal opossum pups attached to the mother's abdomen at P0. **D** Opossum pup at P17. **E** Adult, fully grown opossum. Source: (**A**) *https://en.wikipedia.org/wiki/Gray_short-tailed_opossum* (**B**, **C**) *Samollow 2008* (**E**) *https://www.flickr.com/photos/wcdumonts/15973562891*

Opossums pups are born very immature, at an embryonic stage of development, after a gestational period of 14 days (17). Day of opossum birth, a.k.a. postnatal day 0 (P0, Fig. 1, C) is equivalent to embryonic day (E) 13-14 in rats. In *M. domestica*, peak cortical neurogenesis occurs between P14 and P24 (Fig. 1, D), eyes begin to open at P33, weaning occurs at P56 and opossums reach sexual maturity 5 months after birth (Fig. 2) (18).

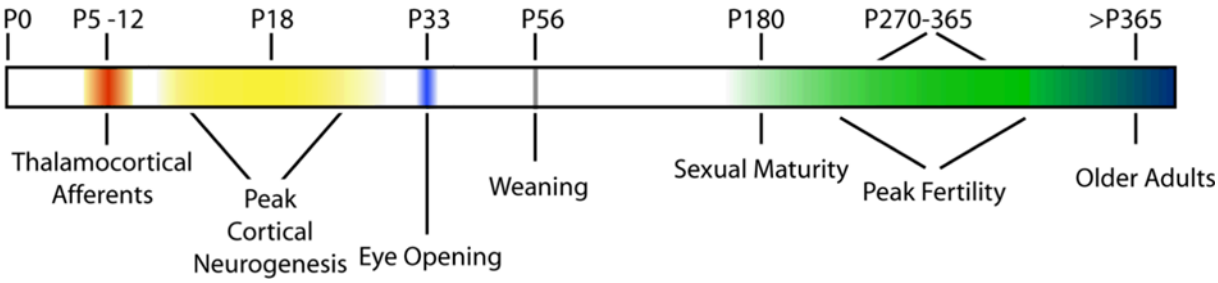


Figure 2. Timeline of significant developmental stages in the life of *Monodelphis domestica*. Important stage is Peak Cortical Neurogenesis (yellow) that happens to occur between P14-24. For spinal cord there are less data available. Source: *Seelke, Dooley, and Krubitzer 2013*

Neonatal opossums were already proven to be useful for developmental studies. The gray opossum is a particularly suitable marsupial for experimental purposes because it is small, easy to maintain and breeds in sufficient number throughout the year, in captivity. Furthermore, thanks to the lack of a pouch, the neonates are accessible for experimental manipulation at a very immature stage and *ex utero* (Fig. 1, C). In comparison, to access eutherian embryos of the same stage of development, the uterus of the pregnant rat or mice needs to be opened (17). There are also many other reasons why opossum is an appealing animal, especially for regeneration studies.

Firstly, in the neonatal opossum, the CNS can regenerate after injury in a short period of two weeks after birth. After that period, the regenerative capacity is abruptly lost. Therefore, like every other mammal, the adult

opossum is not able to regenerate its CNS. Although the exact transition point has not been determined yet, different studies have provided us a narrow window of time when regeneration stops (7). Wintzer et al. (19) have shown that at P9 spinal cord can regenerate after injury, but at P12 regeneration is not possible in cervical segments. Moreover, Saunders et al. (20) showed that in P4 opossum 1-2 weeks after SCI nerve fibers grow through the lesion and the conduction of action potential is restored. In opossums at P14 there was no recovery of function noted. Operations were performed *in vivo* and the crush was made at cervical segment using forceps. In the adult opossums that were operated on at P4, the spinal cord appeared oddly normal, with morphological structure the same as in the non-injured opossums (controls).

Interestingly, because of the rostro-caudal gradient of development, regeneration does not stop in all segments of the spinal cord at the same time. Therefore, in more mature cervical segments regeneration stops at P12, but in the less mature lumbar segment regeneration remains possible until P17 (21). The existence of two differently developed parts in the spinal cord at P12 allows us to compare tissue that can and cannot regenerate in a single animal (19).

Secondly, the isolated CNS of neonatal opossum survives well *in vitro* for days or even weeks. Because the neonatal brain and spinal cord are so tiny, after they are dissected out entirely, they are easy to maintain and they survive very well in culture. In isolated preparations the structure is preserved, functions and development continue apparently normally, all with minimal changes in cell survival. The intact opossum CNS *in vitro* preparation allows monitoring of the CNS development for up to 4 days in Krebs' fluid (22) or up to 10 days in Eagle's basal medium (BME), containing 0.2% fetal calf serum (FCS) and antibiotics (23).

Thirdly, *Monodelphis domestica* was the first marsupial species chosen for genomic sequencing because of its utility for research purposes. Until a few

years ago, all sequenced mammalian genomes were from eutherians. Eutherians (placentals), metatherians (marsupials) and prototherians are the three major groups of modern mammals. Metatherians and eutherians had their last mutual ancestor approximately 180 million years ago, whereupon the lineage diverged from eutherian mammals. Although these two lineages share many fundamental characteristics, they have also developed distinctive features (24). The organization of the neocortex is more complex in eutherian mammals than in the grey opossum (25). The genome sequence of the grey opossum revealed that of the protein-coding sequences which are conserved among eutherians, only ~1% is absent in the opossum. Accordingly, comparative analysis of metatherian and eutherian genome can lead to improving our understanding of the evolution of mammalian genomes, as well as determining key differences in their CNS (24).

Finally, *in vitro* preparations have shown that regeneration is a feature of immature mammalian CNS, rather than some intrinsic feature of the marsupials. The exceptional nature of the opossum, in comparison with mouse or rat, resides in highly immature developmental stage at the birth and longer time-course of postnatal CNS development. By studying the differential gene expression in opossums of different regenerative capacity, our understanding of molecular mechanisms behind promoting or inhibiting regeneration will be expanded. The existence of the narrow window of time when regeneration stops is very useful for studies of differences in marker expression. Differences in expression of particular markers can be examined at different age, at P5 when CNS can regenerate and P16 when regeneration is not possible anymore.

1.3 Primary cell cultures

Primary cell cultures provide an excellent *in vitro* platform for a variety of biochemical, cellular and molecular biological studies under controlled conditions. They are used to examine cellular morphology, proliferative capacities, functions, and signaling, but they also permit easier genetic manipulations (26).

The reason why primary cell cultures are such a major tool in biomedical research is that *ex vivo* cultures retain most of the physiological properties of the tissue they are taken from. Compared to cell lines, primary cell cultures provide more relevant results. Primary cell cultures are heterogeneous[1] and they have a limited lifetime *in vitro*. Different types of primary cells are used in biomedical research, including fibroblasts, epithelial cells, endothelial cells, hematopoietic cells and many others (27).

Due to the complexity of the brain, which makes *in vitro* manipulations a real challenge*,* primary neuronal cell cultures have been mainly developed to study neurons in order to improve our understanding of the CNS molecular and cell biology (28). However, the establishment of primary neuronal culture represents an enormous challenge since mature neurons cannot undergo cell division and they do not proliferate (19). Many well-established primary cell culture techniques are commonly used for the study of cortical neurons from mice (28) and rats (29). However, the brain is not the only source for isolation and culturing of neurons. Seybold and Abrahams (30) managed to establish two different protocols for primary cultures of rat spinal cord, one for neurons and the other enriched in non-neuronal cells.

To our knowledge, dissociated primary neuronal cultures from opossums have not yet been established. Once established and characterized, these cultures could allow the performing of numerous tests on cells in simpler

[1] eng. *heterogeneous* - composed of parts of different kinds; composed of different kinds of cell types which are present in the tissue

environment than *in vivo*, which makes them accessible for observations and manipulations. By using opossum primary neuronal cell cultures for regeneration studies it will be possible to obtain insight into the functional role of key molecules which are involved in CNS regeneration. Since they are relatively easy to manipulate, these cultures allow measuring of the effect of candidate genes by over-expressing them or blocking their expression by gene silencing methods (10).

1.4 Neural stem cells

Stem cells are present in many different tissues, and they are defined by the capacity for long-term self-renewal[2] and potency[3] (31). Neural stem/progenitor cells (NSC) can be derived from embryonic stem cells (ESC), which are pluripotent and have the ability to differentiate into any cell type. In comparison to ESCs, NSCs are a more specialized, tissue-specific subtype.

NSCs are multipotent and self-renewing cells that give rise to multiple types of cells in the CNS through the processes called neuro- or gliogenesis, depending on the cell type generated. Except from ESCs, NSCs can be also derived from induced pluripotent stem cells (iPSC), adult CNS or isolated directly from fetal tissue. In the embryonic CNS they can be found in the neural plate and the ventricular zone (VZ). It has been shown that a limited number of NSCs persist even into adulthood and they continue to produce neurons through life (32). Adult NSCs are found in highly organized regions often referred to as niches. Niches are structures that allow the persistence of a pool of stem and progenitor cells, as well as maintenance of cellular interactions and signaling pathways (33). In the brain, adult NSCs are present in the subventricular zone (SVZ) and the subgranular zone (SGZ) of the dentate gyrus in the hippocampus (32). Furthermore, in the adult

[2] eng. *self-renewal* - division with maintenance of the undifferentiated state
[3] eng. *potency* – the ability to give rise to many types of differentiated cell

rodent spinal cord, NSCs are situated mainly in the ependymal region, meaning the central canal. Ependymal cells surround the central canal and show properties of NSCs. About the spinal cord neurogenic niche much less is known (33). Research studies have shown that NSCs isolated at different developmental stages have in common expression of certain molecular markers, for instance, Nestin and SOX2 (32).

In addition to the ability of self-renewal and differentiation into neurons and glia, NSCs from embryonic and adult CNS have another common property: the ability to form neurospheres. Neurospheres are floating spheroid aggregates of NSCs which are formed by exposing dissociated cells from tissue to growth factors and used to examine characteristics of NSCs.

Cultured NSCs provide a research tool for study of CNS development, structure and function. NSCs can be used as a source of all types of cells present in the mammalian CNS, including radial glial cells, glial cells and neurons. Moreover, NSCs represent valuable therapeutic tool. Different studies can be performed on the cells deriving from NSCs *in vitro* in order to elucidate the molecular basis of neural disease as well as potential therapeutic strategies (31). Although much is known about stem cells in the mammalian brain, less attention has been given to those in the spinal cord.

1.5 Neural lineage development and markers

The cellular composition of the CNS is fairly complex and, unfortunately, still not completely understood. Identification of cell types present in the CNS has been a challenge for scientists all over the world.

The CNS develops from the ectoderm, one of the three germinal layers. The early mammalian CNS initiates as a neural plate that undergoes neurulation to form the neural tube, initially open at both ends. The tube

rapidly differentiates and both ends expand. The rostral end develops into the brain and the caudal into the spinal cord. Also, the neural crest, which gives rise to the sensory and autonomic neurons and Schwann cells, is formed by a small group of neural fold cells (34). The population of neural progenitor cells (NPC) is heterogeneous and insufficiently characterized. Timing of neurogenesis and types of NPCs are quite different between different species as well as throughout CNS regions. NPCs, as a heterogeneous population, may differ in their morphology, division and lineage they generate (35).

During early embryonic development, the neural plate and neural tube are composed of proliferative neuroepithelial cells (NEC). NECs are present in the CNS before neurogenesis begins, thus they represent the earliest progenitors in the CNS. The characteristic feature of NECs is their bipolar shape and the expression of Nestin and SOX2 (Fig. 3) (36).

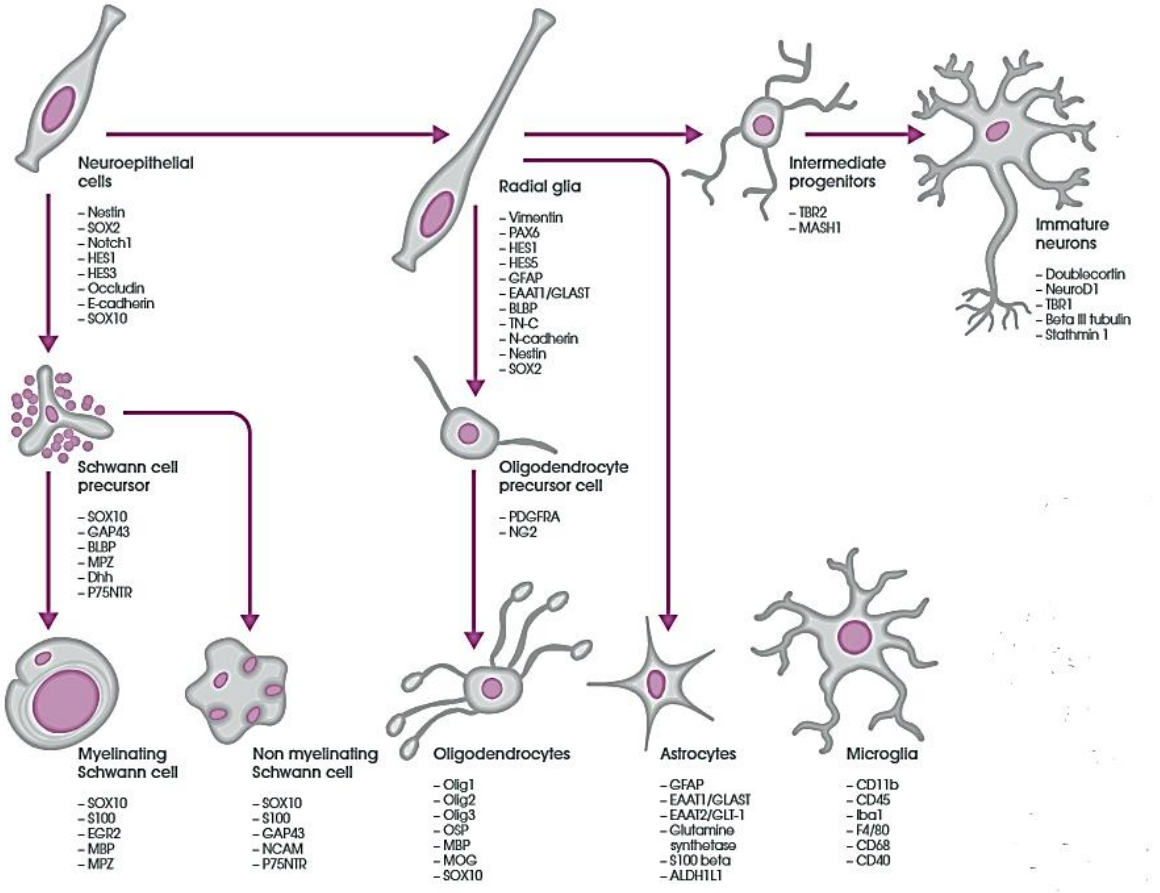


Figure 3. Schematic representation of the main cell types that are present in the CNS and their corresponding molecular markers.

Source: *http://docs.abcam.com/pdf/neuroscience/neural-lineage-markers-web.pdf*

NECs undergo self-renewing symmetric division and in that way expand the size of the precursor cell pool while forming the neural plate (35). Simultaneously, at the later stages of development, NECs start to divide asymmetrically producing one identical NEC and one basal progenitor (BP). BPs can also be generated by radial glial cells (RGCs), which will be explained later in this chapter. In this thesis, BPs derived from RGCs are referred to as intermediate progenitor cells (IPCs). It is important to note that, regardless of their different origins and names, the BPs and IPCs are identical cells. BPs divide symmetrically and generate two identical daughter cells that exit the VZ and differentiate into neurons. BPs form a layer known as the SVZ.

Around the onset of neurogenesis (Fig. 4), NECs start to change their molecular features and switch their identity into RGCs. It is important to note that the exact time point of the appearance of RGCs is still unknown and debatable. Multipotent RGCs are located in the VZ and they can be distinguished from NECs by the expression of astroglial markers, such as BLPP, PAX6 or GFAP (Fig. 3). RGCs are considered to be the main neural progenitors that have the potential to generate all the main lineages of CNS: neurons, oligodendrocytes, astrocytes, adult NSCs, and ependymocytes. Furthermore, they have an important role in supporting the migration of newly generated neurons (36).

Radial glial fate and progeny differs intrinsically between CNS regions, particularly between the VZ and the SVZ. RGCs undergo both symmetric and asymmetric division. At first, RGCs divide symmetrically in order to expand the quantity of proliferative population in the VZ. Later, RGCs begin undergoing asymmetric divisions, and the result is the generation of one self-renewed RGC and a neuronal daughter cell (Fig. 4). NPC daughter cell migrate to the SVZ and is called IPC or BP. This two-step pattern of neurogenesis makes it possible to simultaneously increase the number of neurons of a particular subtype and generate neuronal diversity (37).

IPCs are located in the SVZ and show expression of Tbr2 (Fig. 3), a T-domain transcription factor. Unlike RGCs, IPCs do not express PAX6. IPCs have a limited capacity for self-renewal and are exclusively neurogenic– by symmetric division in the SVZ they generate a pair of neurons. This transition to post-mitotic, immature neurons is characterized by downregulation of the progenitor marker Tbr2 and upregulation of Tbr1 (38). In addition to Tbr1, immature neurons also express β-tubulin III (Fig. 3).

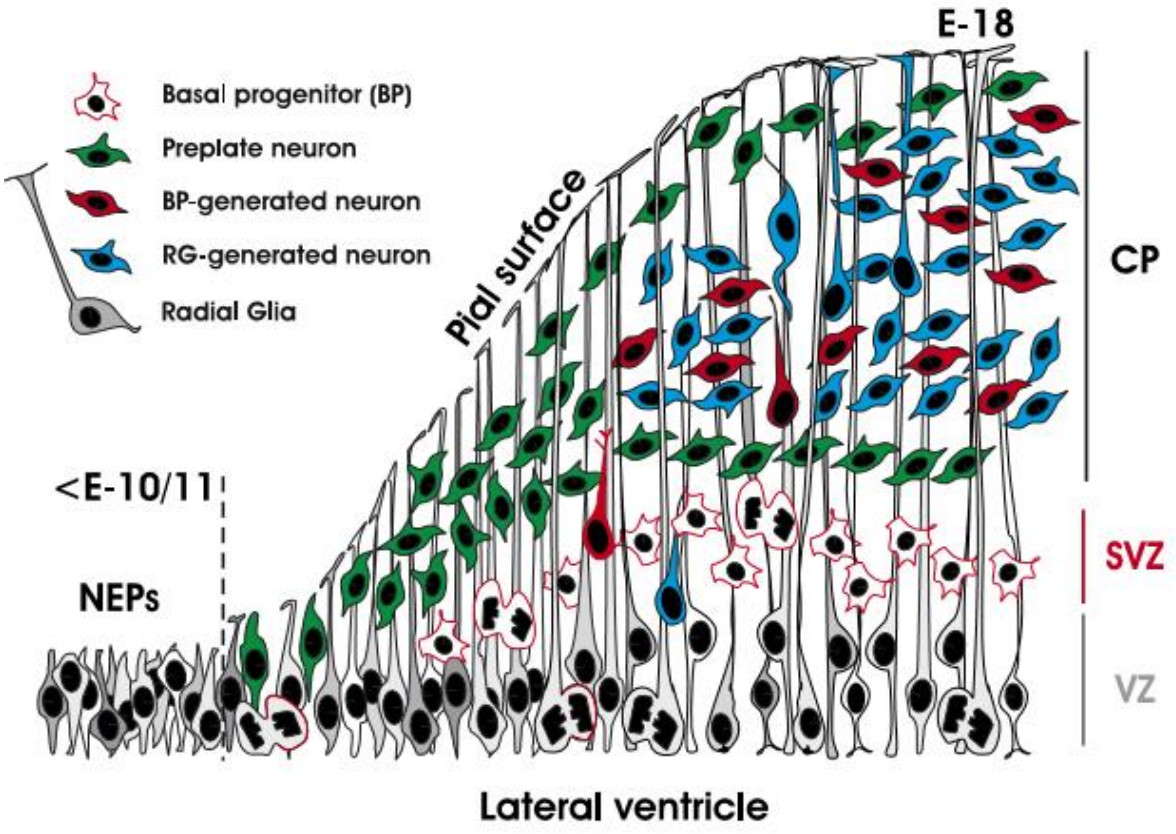


Figure 4. The early stage of neurogenesis – a transition from NECs to RGCs. Around the onset of neurogenesis, NECs switch their identity into RGCs. Basal progenitors (BP, red outline) originate from NECs at early stages and later from RGCs. BPs form the SVZ and generate preplate neurons (green). Later in neurogenesis, neurons are generated both from BPs (red) and RGCs (blue). Abbreviations: NEPs, neuroepithelial progenitor cells; VZ, ventricular zone; SVZ, subventricular zone; CP, cortical plate. Source: *Malatesta, Appolloni, Calzolari 2007.*

1.5.1 NSC markers

The SOX family is a highly conserved group of transcriptions factors characterized by homology to the high-mobility-group (HMG) domain that mediates DNA binding. The HGM domain was first identified in SRY, a gene involved in mammalian male sex determination. Therefore, SOX stands for SRY-related HMG box.

There are 20 SOX proteins identified in humans and mice that are grouped in 9 subgroups (Fig. 5, A) based on the homology they share outside the HGM domain (39). The SOX proteins of interest for this thesis are SOX2 and SOX9.

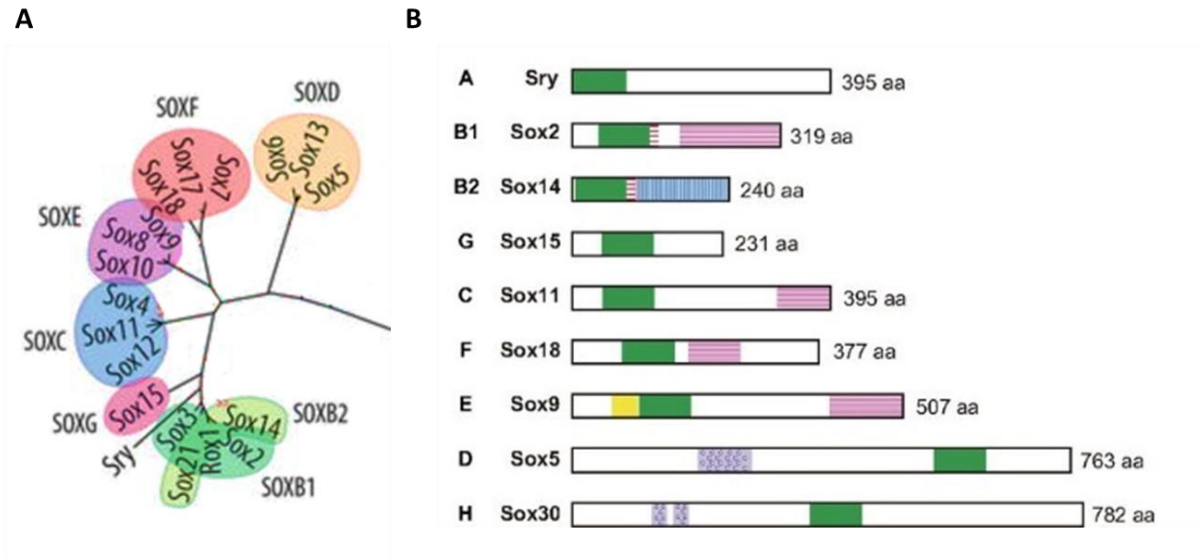


Figure 5. Grouping of SOX proteins and schematic structures of representatives of each group.

A The phylogenetic tree of the SOX proteins divided into 9 subgroups. All SOX2 proteins have HMG domain, but they are divided into subgroups based on homology they share outside the DNA-binding HGM domain. **B** The schematic structures of mouse Sox protein domains. Different colors mark different important domains. The DNA-binding HMG domain is highly conserved among members of each group (green). There is also a transcriptional activation domain (pink), transcriptional repression domain (blue), dimerization domain (yellow). Horizontal red stripes in B1 and B2 group represent group B homology. Source: *(**A**) Vizoso-Vázquez et all 2017. (**B**) Kamachi and Kondoh 2013.*

1.5.1.1 SOX2

SOX2 is a transcription factor that, along with SOX1 and SOX3, constitute the SOXB1 subgroup. SOX2 is well known as a stemness and pluripotency factor and it is considered to be a universal stem cell marker at all stages of CNS development (32).

SOX2 has a key role in maintaining the undifferentiated precursor state, meaning that its presence inhibits premature neuronal differentiation and promotes proliferation. Hutton and Pevny (40), have shown that NPC populations can be distinguished by expression levels of SOX2. Accordingly, in RGCs SOX2 is highly expressed, while the level of expression is lower in IPCs. RGCs have the capacity to give rise both to neurons and glial cells, while IPCs generate only neurons. Even after specification into the glial lineage, SOX2 remains expressed in astrocytes and oligodendrocytes through embryonic development (41). On the other hand, it is downregulated in post-mitotic neurons.

Moreover, all neurospheres in the CNS are generated by SOX2 expressing cells. An interesting finding is that the size, self-renewal and pluripotency factor of neurospheres correlate with increased levels of SOX2 expression. Regarding the size, small neurospheres generate only neurons while neurospheres with large diameters generate both glia and neurons (40).

1.5.1.2 SOX9

SOX9 is a transcription factor classified into the SOXE subgroup, along with SOX8 and SOX10. It was first identified as a gene underlying Campomelic dysplasia[4] (39). Today, it is known that SOX9 regulates broad aspects of development both in CNS and in the neural crest (42).

Although the exact mechanism that induces NSCs to switch from neurogenesis to gliogenesis is still unknown, SOX9 is considered to be an

[4] eng. *Campomelic dysplasia* (CD) – human haploinsufficiency disease featured by high proportion of male sex reversal

important molecular component in this process. During embryonic development of the CNS, SOX9 is expressed by multipotent NSCs and glial progenitors. It has an important role in driving the specification of the glial lineage, which includes two types of cells in the CNS: oligodendrocytes and astrocytes (43).

Another study has determined SOX9 to be an astrocyte-specific nuclear marker in the adult brain (42). Astrocytes have a diverse role in CNS, such as modulating neuron activity, clearing metabolic waste products, and forming the blood-brain barrier. For their survival and normal function, neurons depend on a symbiotic relationship with astrocytes. Astrocytes that express SOX9 make up ~10-20% of the total number of brain cells. Moreover, the same study revealed that SOX9 expression does not change during aging, but it is upregulated by astrocytes in the number of different conditions, such as stroke and amyotrophic lateral sclerosis (ALS) and importantly, after SCI.

1.5.2 Neuronal marker

1.5.2.1 ß - Tubulin III

Microtubules are the major component of the eukaryotic cytoskeleton, therefore, they have a role in many cellular processes, including mitosis, meiosis, and maintenance of cell structures. They are assembled of many basic structural subunits called tubulin heterodimers. Tubulin consists of two structural components - α-tubulin and β-tubulin.

β-tubulin III is one of the seven β-tubulin isotypes identified in the human genome, and it is encoded by TUBB3. Unlike other classes of tubulin that are mainly involved in cellular processes like mitosis, β-tubulin III is considered to be expressed specifically during the differentiation of neurons. Accordingly, results obtained by immunocytochemical staining with anti-β-tub III showed that it is found in cell bodies, axons, and

dendrites of immature neurons (44). In different studies, β-tubulin III was used as a neuron-specific marker of newly generated postmitotic neurons, but it can also be found in some NPCs that are mitotically active (45). Glial cells also express isotypes of ß-tubulin, but antibodies against β-tubulin III do not recognize them and therefore are specific for neurons.

2. Aim of the thesis

Primary cell cultures represent a powerful *in vitro* tool because, unlike immortal cell lines, they maintain many of the functions and express important markers observed *in vivo* (26).

In the previous research conducted by the Laboratory for Molecular Neurobiology, one of the objectives was to prepare opossum spinal cord primary cell cultures from animals of different age that either can (P5) or cannot (P16) regenerate after SCI. Cortical primary cultures were established as well. Since dissociated primary neuronal cultures from opossums have not yet been established, the extensive characterization with cell type-specific markers is necessary.

We hypothesized that differences in expression of markers in primary neuronal cultures from opossums of different regenerative capacities may provide an important insight into the key molecules that might have an important role in promoting or inhibiting regeneration. Therefore, the aim of this thesis was:

1. to characterize opossum cortical and spinal cord primary cell cultures using standard cellular markers for stem/progenitor, neuronal and glial cells
2. to test different cell culture media in order to favor either proliferation and expansion of the neural stem cells or neuronal differentiation and survival
3. to characterize opossum fresh-fixed spinal cord tissue using standard cellular markers for stem/progenitor, neuronal and glial cells
4. to analyze and compare the results obtained with immunocytochemistry and immunohistochemistry

3. Materials and methods

3.1 Primary neuronal cultures

The opossum (*Monodelphis domestica*) colony was hosted at the animal house facility of the University of Trieste. There, the animals were maintained in accordance with the guidelines of the Italian Animal Welfare Act, and their use was permitted by the Ethics Committee board, the Local Veterinary Service, and the National Ministry of Health (Permit Number: ADD) in accordance with the European Union guidelines for animal care (d.1.116/92; 86/609/C.E.). All the experiments described in this thesis using opossum were conducted in accordance with European Directive 2010/63/EU for animal experiments. Throughout the dissociation procedure, all efforts had been made to minimize both animal suffering and the number of animals used.

The dissociation procedure was developed following an already established protocol for the postnatal mouse (Beaudoin et al., 2012) (28) with a few modifications. All the instruments used for dissection were precleaned with ethanol (EtOH, Gram-Mol) or 1% Incidin (Ecolab). Two days before plating, 12mm coverslips were washed in 1M hydrochloric acid (HCL, Normapur) overnight at room temperature (RT). The following day HCL was removed, coverslips were washed 3x with distilled water (dH2O or Milli-Q) and then with absolute EtOH for an hour at RT. After removing the EtOH, coverslips were spread in a glass Petri dish (ISOLAB) and dry sterilized in the oven at 150 °C for 90 minutes. The sterile coverslips were placed in the wells of a 24-well tissue culture plate (Sarstedt), each coverslip was covered with 70 µL of 50 µg/mL poly-L-ornithine (Sigma-Aldrich), the plate was wrapped with parafilm and incubated overnight at 32°C. The next day poly-L-ornithine was removed from coverslips which were then washed 2x with sterile H_2O and left in the laminar for 15 minutes to dry. Onto the dry coverslips was put 60 µL of laminin (Sigma-Aldrich) solution diluted 1:1000

(1-2 µg/mL) for cortical and 1:200 (5-10 µg/mL) for spinal cord cell cultures.

Opossum at different postnatal ages, P5 and P16, were killed by decapitation with scissors. The dissection of the cortex and spinal cord was performed in dissection solution cooled down to 4°C, under a dissecting microscope with illumination. The dissection solution consisted of Hanks' Balanced Salt Solution (HBSS) without Ca^{2+}/ Mg^{2+}, 3.5 g/L glucose (Merck), 10mM of hydroxyethyl-piperazine-ethane-sulfonic acid (HEPES), 3% bovine serum albumin (BSA) and 1% Penicillin/Streptomycin (all three from PAN Biotech). After removing the meninges from cortex and the spinal cord, the tissues were put separately in the 2 mL tubes, washed 3x in phosphate buffer saline (PBS, Merck) and chopped into tiny pieces using a scalpel.

The next step was enzyme digestion in which the digestion medium was used to relax the tissue structure and permit better separation of the cells. Because the cortex is rather different from the spinal cord, we established different protocols for cortex and spinal cord enzyme digestion. The digestion medium for the cortex tissue consisted of 0.5 mg/mL trypsin in Dulbecco's Modified Eagle Medium (DMEM, all from PAN Biotech), while in the enzyme mix for the spinal cord tissue 1 mg/mL collagenase (Sigma-Aldrich) was added. Also, the incubation time was different depending on the tissue and the age. The cortices of P5 pups were incubated in the digestion medium for 10 minutes, while those of P16 pups were incubated for 20 minutes. The P5 spinal cord was incubated in the enzyme mix for 20 minutes, while of P16 was incubated for 30 minutes. All incubations were in a tube in a thermo block at 32°C, because that temperature corresponds to the mean basal opossum's body temperature (46).

Next, the samples were washed 3x in PBS and resuspended in triturating solution composed of dissection solution enriched with 10% fetal bovine serum (FBS, PAN Biotech, Germany). Both tissues were triturated mechanically by pipetting using a 1mL tip at least 15x in order to break the

tissue structure. Cortex from the P17 opossum was triturated 3x as the tissue is bigger and firm. The supernatant was collected and dropped onto a BSA cushion (4% BSA in HBSS) in a 5 mL tube and then and pipetted once more until no tissue was visible.

The cells were centrifuged for 10 minutes at 1000 revolutions per minute (rpm) at RT and resuspended in 2 mL of plating medium composed of 5% FBS in neuronal medium. Neuronal medium or medium for neuronal differentiation was composed of 1% Penicillin/Streptomycin, 1% stable glutamine (all from PAN Biotech, Germany), and B27 supplement in Neurobasal medium (Thermo Fisher Scientific).

The cells in the plating medium were preplated in a 30mm plastic Petri dish (Sarstedt) and incubated for 10 minutes in the incubator at 32°C. Preplating was the additional step that allowed adherence of fibroblasts and consequently their removal from the cell suspension.

Cells were collected from the Petri dish into a 15 mL tube (Greiner), diluted with the proper volume of plating medium to get the 100 000 cells per well (final volume of 0,5 mL per well) and plated in a 24-well tissue culture plate on coverslips previously coated with poly-L-ornithine and laminin. After one day 2/3 of the plating medium was changed with fresh neuronal medium. The neuronal primary cultures were maintained in an incubator at 32ºC in 5% CO_2 and 95% relative humidity for 1 or 7 days before the immunofluorescence was performed.

To test the impact of different cell culture media, one modification was added to the protocol for primary neuronal cultures – different plating medium.

3.1.1. Primary neuronal cultures in neuronal medium for differentiation

Neuronal plating medium for neuronal differentiation was composed of 10% FBS in Neurobasal medium with B27 supplement, 1% Penicillin/Streptomycin and 1% stable glutamine. After one day 1/2 of the plating medium was changed.

3.1.2. Primary neuronal cultures in glial medium for proliferation

Glial (stem cell) plating medium for proliferation and expansion of the neural stem cells was composed of 10% FBS in DMEM with stable glutamine, 4,5 g/L glucose, 3,7 g/L sodium bicarbonate ($NaHCO_3$) and 1% Penicillin/Streptomycin. After one day 1/2 of the plating medium was changed.

3.2 Immunofluorescence

Immunofluorescence (IF) is a generally used laboratory technique that allows visualization of the presence, location, and distribution of a target antigen in a sample. IF is based on the use of specific antibodies which have been chemically conjugated to fluorescent dyes. Target antigens interact with specific antibodies tagged with fluorophores and as a result, became visible. Depending on the biological sample that is analyzed we differentiate between immunocytochemistry (ICC) and immunohistochemistry (IHC).

ICC is performed on samples of whole cells removed from the extracellular matrix. Samples can be derived from any suspension of cells, obtained from the animal or cultured cells grown in a monolayer on coverslips. This technique allows visualization of specific antigens in isolated cells.

IHC is done on samples of biological tissue removed from the animal and then histologically processed into thin sections which are mounted onto slides. This technique allows visualization of specific antigens within cells while preserving the original architecture of the surrounding tissue.

3.2.1. ICC

Cell medium was aspirated and then coverslips, containing cells from cortex or spinal cord, were washed 2x with 1xPBS and fixed in 500μL 4% paraformaldehyde (PFA, Millipore) for at least 20 minutes at RT. Fixation is applied with the aim of preserving cellular structure, that is, to

retain the cells or tissue in a state as near to life as possible by quickly terminating all metabolic activities to minimize post-fixation changes.

After fixation, cells were washed 3x in 1xPBS, saturated with 0.1 M glycine (Sigma-Aldrich) for 5 minutes, washed again with 1xPBS for 5 minutes and permeabilized with 0.1% Triton X-100 in PBS (all from Merck, Germany) for 10 minutes. Blocking was done with 0.5% BSA in PBS for 30 minutes, and then cells were incubated with primary antibodies diluted in PBS (Table 1). Incubation lasted for 1h in the dark wet chamber, where 100 µL of primary antibody solution was put on face-up coverslips, placed onto the parafilm layer. The primary antibody was omitted when performing negative controls.

After the incubation, coverslips are put back into the dish, washed 2x with 1xPBS for 5 minutes and then returned in the dark wet chamber where, as before, were incubated with secondary antibodies diluted in PBS (Table 2). Besides the secondary antibodies, into the solution was added the nuclear stain 4',6-diamidino-2-phenylindole (DAPI, 1:100, Thermo Fisher Scientific) and cells were incubated for 45 minutes. All the incubations were performed at RT.

Afterwards, coverslips are put back into the dish, washed 2x with 1xPBS and then washed with dH_2O for 5 minutes. As a final step, coverslips were mounted. A small drop of mounting medium (Vectashield, Vector Laboratories) was added on the microscope slide (ISOLAB), the coverslip was put on the drop face down and sealed with nail polish. Slides were left to dry for an hour at RT whereupon were analyzed using a fluorescent microscope or stored flat at 4 ºC protected from light.

3.2.2. IHC

Prior to IHC staining, spinal cord is removed from the opossum and fixed by immersion in 4% PFA at 4 ºC for 12-24 hours. After the fixation, PFA is removed and the tissue is washed 2x with 1xPBS. At least 3 hours before cutting the tissue needs to be immersed in 30% sucrose in

PBS, which is a common cryoprotectant used to preserve tissue morphology by preventing ice crystal formation in tissues when water freezes and expands.

Afterwards, the spinal cord was cut, and the lumbar section was isolated, embed in optimal cutting temperature (OCT) medium Killik (Bio Optica Milano s.p.a.) avoiding bubbles and frozen at -80 ºC. Frozen OCT containing the tissue was put onto specialized metal grids that fit on the cryostat and the tissue is cut on 16 µm thick slices at -30 ºC. The slices collected on microscope slides (Thermo Fischer Scientific) are dried for 30 minutes at RT. At this point, slides can be stored at -20ºC or prepared for IHC staining.

IHC staining runs over two days. The first step on day 1 was to trim the slides with hydrophobic pen (Vector Laboratories, Inc), whereupon slides were washed 2x for 5 minutes in 0.1% Tween 20 (Applichem) in 1xPBS (PBS-T), and then 0.1M glycine was added for 10 minutes. For blocking, blocking solution was used made from 3% normal goat serum (NGS, PAN Biotech), 3% BSA, 0.3% TritonX-100 and 10x PBS. Blocking was incubated for an hour at RT. Primary antibodies (Table 1) were diluted in antibody solution made from 1% NGS, 1% BSA, 0.1% Tween 20 and 10xPBS, added on slides and incubated in humid chamber overnight at 4ºC. The primary antibody was omitted when performing negative controls.

The next day slides were washed 3x with PBS-T for 5 minutes, and then incubated with secondary antibody (Table 2) and nuclei stain DAPI (1:100) diluted in antibody solution, in a humid chamber for 2 hours at RT. After incubation slides were washed 2x for 5 minutes in PBS-T and the final wash was done in dH2O. The excess water from slides was dried, the two drops of mounting medium per slide were added and the 24x60 mm coverslip (Thermo Fischer Scientific) was put onto the slide. The coverslips were sealed with a thin layer of nail polish. Slides were left to dry for half an hour at RT whereupon were analyzed using a fluorescent microscope, stored short-term at 4 ºC or long term at -20 ºC, protected from light.

Table 1 Primary antibodies

Primary (1◦) antibody	Host and type	Dilution	Immunogen	Manufacturer Catalog # Lot #
Anti - SOX2 antibody [9-9-3]	Mouse monoclonal IgG1	ICC 1:200 IHC 1:500	Synthetic peptide conjugated to KLH derived from within residues 300 to the C-terminus of Human SOX2	Abcam ab79351 Lot: GR3202055-1
Anti - SOX9 antibody [EPR14335-78]	Rabbit monoclonal	ICC 1:200 IHC 1:500	Recombinant fragment within Human SOX9 aa 150-300 (internal sequence)	Abcam ab185966 Lot: GR3194558-1
Anti –ß-Tubulin III	Rabbit polyclonal	ICC 1:200	Synthetic peptide corresponding to aa residues 441-450 of human β-tubulin III (Ala 446 to Ser446 substitution) with N-terminal added cysteine, conjugated to KLH	Sigma-Aldrich T2200 Lot: 037M4851V
Anti-β-Tubulin Isotype III antibody	Mouse monoclonal IgG2b	ICC 1:200	Peptide corresponding to the carboxyl-terminal sequence of human β-tubulin isotype III, conjugated to BSA	Sigma-Aldrich T5076 Lot: 056M4864V
Beta Tubulin Antibody	Mouse monoclonal IgG2a	ICC 1:200	Beta Tubulin fusion protein Ag0117	Proteintech 66240-1-Ig Lot: 10004491
Anti-Tubulin β 3 (TUBB3) Antibody (TUJ1)	Mouse monoclonal IgG2a	ICC 1:200	Microtubules derived from rat brain	Biolegend 801201
Anti - NeuN antibody [EPR12763]	Rabbit monoclonal	ICC 1:200 IHC 1:500	Synthetic peptide within Human NeuN aa 1-100 (Cysteine residue)	Abcam ab177487 Lot: GR249899-65
Anti – GFAP antibody [EP672Y]	Rabbit monoclonal IgG	ICC 1:200	Synthetic peptide (the amino acid sequence is considered to be commercially sensitive) (C terminal)	Abcam ab33922 Lot GR148838-3

Table 2 Secondary antibodies

Secondary (2◦) Antibody	Host and type	Target species	Dilution	Immunogen	Manufacturer Catalog # Lot #	Conjugation
Goat Anti-Mouse IgG H&L	Goat polyclonal	Mouse	ICC 1:400 IHC 1:500	Not available	Abcam ab150119 Lot: GR3218802-2	Alexa Fluor® 647
Goat anti-Rabbit IgG (H+L)	Goat polyclonal IgG	Rabbit	ICC 1:400 IHC 1:500	Gamma Immunoglobins Heavy and Light chains	ThermoFisher A32732 Lot: TC267122	Alexa Fluor® Plus 555
Goat anti-Mouse IgG (H+L)	Goat polyclonal IgG	Mouse	ICC 1:400 IHC 1:500	Gamma Immunoglobins Heavy and Light chains	ThermoFisher A32723 Lot: TC252656	Alexa Fluor® Plus 488
Goat anti-Mouse IgG1	Goat polyclonal IgG1	Mouse	ICC 1:300 IHC 1:500	IgG gamma 1	ThermoFisher A21121	Alexa Fluor® 488
Goat anti-Mouse IgG2a	Goat polyclonal IgG2a	Mouse	ICC 1:300 IHC 1:500	Mouse IgG2a	ThermoFisher A21137	Alexa Fluor® 555
Goat anti-Rabbit IgG (H+L)	Goat polyclonal IgG	Rabbit	ICC 1:400 IHC 1:500	Gamma Immunoglobins Heavy and Light chains	ThermoFisher A32731	Alexa Fluor® Plus 488
Goat anti-Mouse IgG (H+L)	Goat polyclonal IgG	Mouse	ICC 1:400 IHC 1:500	Gamma Immunoglobins Heavy and Light chains	ThermoFisher A32727	Alexa Fluor® Plus 555
Goat anti-Rabbit IgG (H&L)	Goat polyclonal IgG	Rabbit	ICC 1:400	Not available	Abcam ab150083 Lot: GR3234654-1	Alexa Fluor® 647

3.3 Fluorescence microscopy

Fluorescence microscopy is used to visualize the fluorescence of specific cellular components in as native a state and organization as possible. The staining of samples was analyzed using an Olympus IX83 inverted fluorescent microscope (Olympus, Japan) equipped with a Xenon lamp, Hamamatsu Orca R2 CCD camera (Hamamatsu, Japan), DIC, fluorescence optics, and Cell Sense software (Olympus, Japan). The objective that was mostly used was 20x 0.5NA.

Table 3 The set of filter cubes used for fluorescence microscopy

	Unit Name	Excitation filter	Emission filter	Dichromatic mirror
Ultraviolet Excitations	U-FUNA	360-370	420-460	410
Blue Excitations	U-FBW	460-495	510IF	505
Green Excitations	U-FGW	530-550	575IF	570
Far-Red Excitations	Cy5	590-650	663-738	660

For each image, a Z stack was acquired containing at least 15 slices with slice spacing of 1 μm. Every image was processed with Image J by Wayne Rasband (developed at the U.S. National Institutes of Health and available at http://rsbweb.nih.gov/ij/).

3.4 Statistical analysis

All data acquired by counting using ImageJ plugin Cell Counter were analyzed and plotted in programs the Microsoft Excel and Prism 6.0 software (GraphPad, statistical analysis). Results are presented as mean values with associated standard deviations. The statistical test used was two-way ANOVA and a p-value of $p<0.05$ was chosen for statistical significance.

4. Results

4.1 Cortical primary cell culture

We prepared cortical primary cell cultures from P5 and P16 opossums. The protocol was developed based on a previously established protocol for culturing murine pyramidal neurons (28). The cell cultures were maintained in the incubator for 7 days *in vitro* (DIV). One day after cell plating in neuronal medium with 5% FBS, we observed cell survival and neurite outgrowth by light microscope (Fig. 6, DIV1). The cultures derived from P16 opossums (Fig. 6, bottom row) have a visibly higher yield of cells than cultures from P5. After seven days in culture morphology of neurons developed and neuronal density increased which strongly indicated that *in vitro* neuronal differentiation occurred. Under the light microscope it is

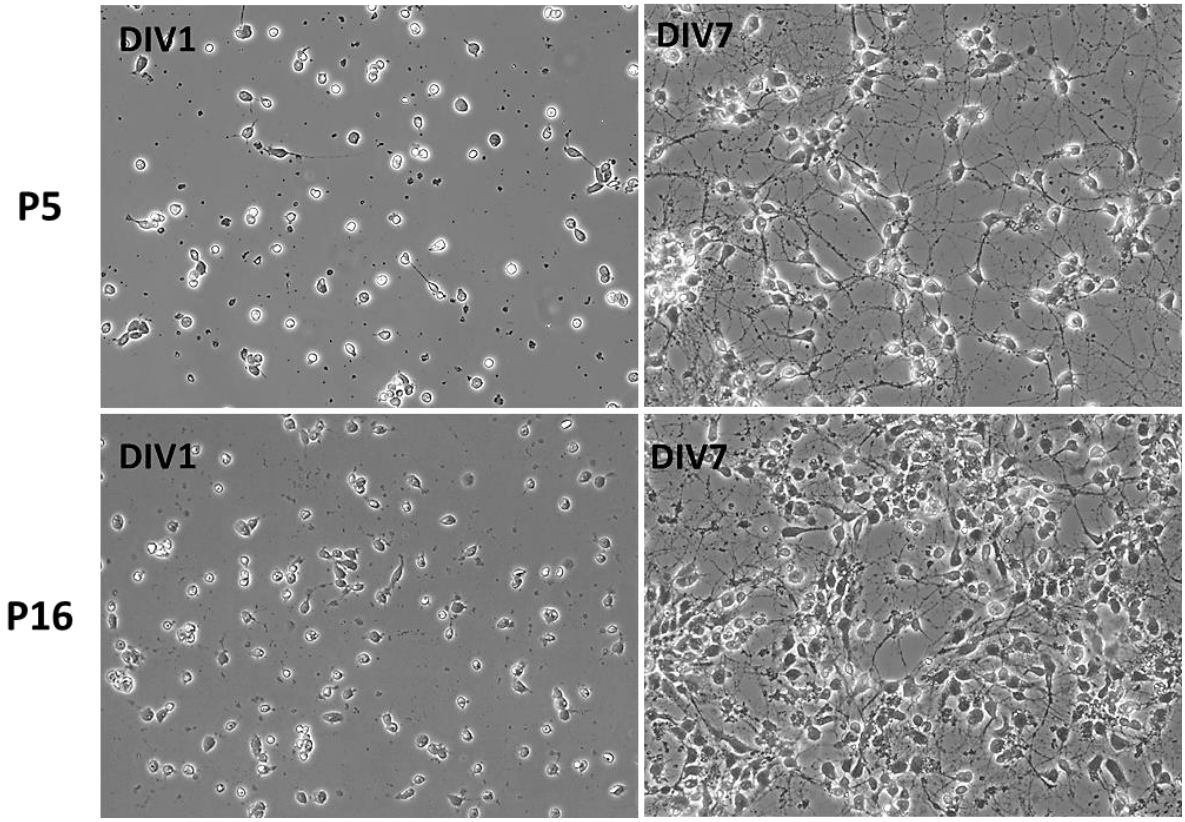


Figure 6. Brightfield images of cortical primary cell cultures from P5 and P16 opossum at two different stages during culture, DIV 1 and DIV7. The cortical primary cells from P5 (upper row) and P16 (bottom row) opossum were plated on coverslips pretreated with poly-L-ornithine and laminin (1:1000) in 24-well tissue culture plate. The cells were cultivated in plating medium composed of 5% FBS in neuronal medium. Cultures were maintained in an incubator at 32ºC in 5% CO_2 and 95% relative humidity. Images of the cultures were taken at DIV1 and DIV7 with camera connected to the light microscope at magnification 20x.

possible to observe more mature neuronal morphology, with developed axons and dendrites (Fig. 6, DIV7).

4.2 Spinal cord primary cell culture

Once we established an optimal protocol for cortical primary cell cultures it was necessary to prepare spinal cord primary cell cultures as previously done in rats by Seybold and Abrahams (30). Spinal cord cultures were plated also in neuronal medium with 5% FBS and maintained in the incubator for 7DIV. At DIV1 we can observe that cultures deriving from P16 have slightly higher yield in comparison with cultures from P5 (Fig. 7, DIV1). Cells are round, well separated and bright. At DIV7 cultures are rich in neurons with long axons, and non-neuronal cells that are more abundant

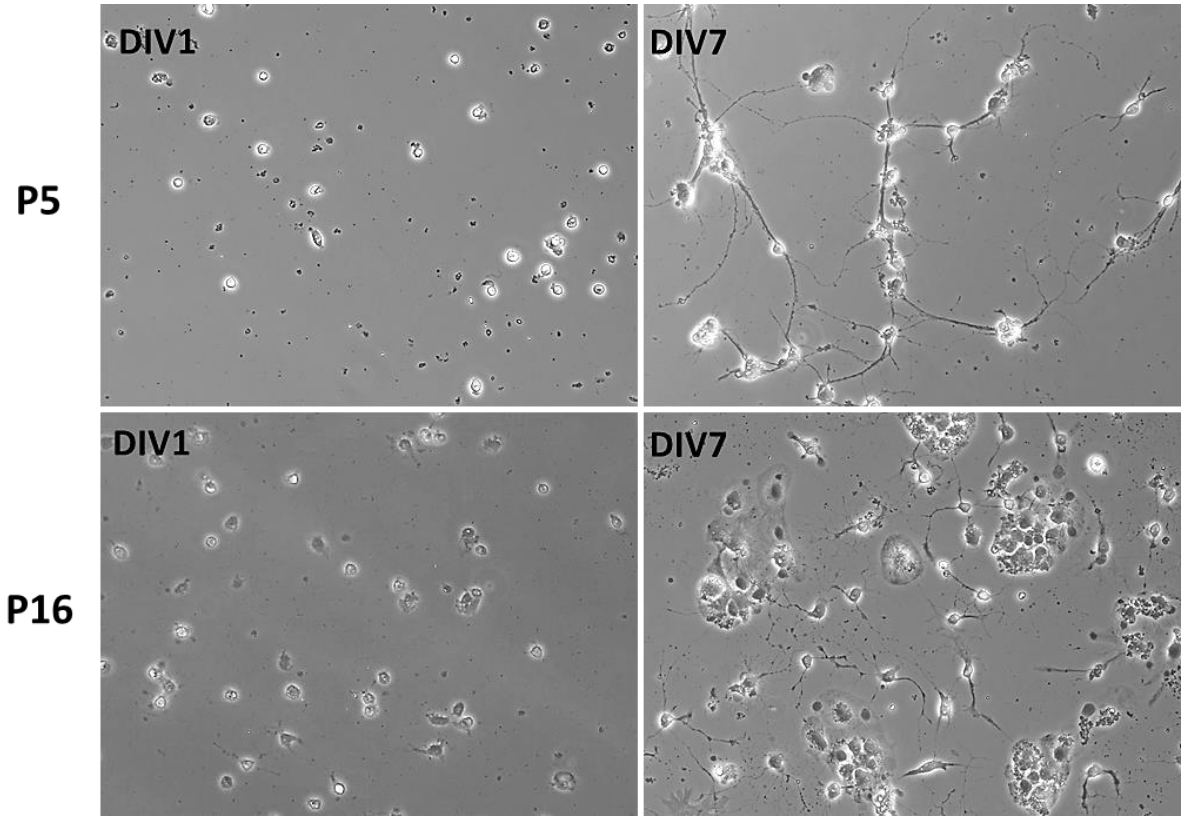


Figure 7. Brightfield images of spinal cord primary cell cultures from P5 and P16 opossum at two different stages during culture, DIV 1 and DIV7. The spinal cord primary cells from P5 and P16 opossum were plated on coverslips pretreated with poly-L-ornithine and laminin (1:200) in 24-well plate. The cells were cultivated in plating medium composed of 5% FBS in neuronal medium. Cultures were maintained in an incubator at 32ºC in 5% CO2 and 95% relative humidity. Images of the cultures were taken at DIV1 and DIV7 with camera connected to the light microscope at magnification 20x.

in P16 (Fig. 7, DIV7). In comparison with cortical primary cell cultures (Fig. 6) these cultures have a smaller yield of neurons. The smaller yield of neurons could be due to the stronger enzymatic treatment required for the effective dissociation of the spinal cord. Alternatively, spinal cord neurons could have a lower survival rate in culture compared to cortical neurons.

4.3 Characterization of opossum cortical and spinal cord primary cell cultures using immunocytochemistry

After successful preparation of the opossum cortical and spinal cord primary cell cultures from animals of different age, the next step was characterization of CNS markers by immunostaining. Markers of interest were the stem/progenitor marker Sox2, stem/glial progenitor marker Sox9, and marker for immature neurons, ß-Tubulin III. Based on marker positivity, we can identify cell types present in cell culture.

Since most antibodies are not validated on *Monodelphis domestica*, first it was necessary to test if each antibody reacts with our chosen species. In our previously conducted experiments, primary antibodies anti-SOX2 and anti-SOX9 have been validated on opossum. These antibodies worked excellent both for ICC and IHC. However, four different primary antibodies for ß-Tubulin III (Table 1) needed to be validated.

4.3.1 ß- Tubulin III antibody optimization

Searching through properties of each antibody against β-Tubulin III, we found that all of them react with different epitopes present on the β-tubulin of human, rat, and mouse. As mentioned previously, β-Tubulin (TUBB3) encodes for the isoform of the microtubule protein tubulin, specifically expressed in neurons.

First, we ran a sequence similarity search of human protein TUBB3 with the Basic Local Alignment Search Tool (BLAST) program and we found the corresponding opossum protein TUBB6. Next, we aligned the human and opossum protein sequences with the *Clustal Omega* program to compare their characteristics. The alignment showed that there are 415 identical residues (of 450) and 92.22% identity (Fig. 8). Based on the identity percentage, we are not able to predict if all antibodies will react with opossum. As mentioned, antibodies react with different epitopes so it is possible that the part of sequence they recognize in human, is not identical in the opossum.

```
F7CB79 F7CB79_MONDO      1   MREIVHIQAGQCGNQIGTKFWEVISDEHGIDPAGGYVGDSALQLERINVYYNESSTQKYV   60
Q13509 TBB3_HUMAN        1   MREIVHIQAGQCGNQIGAKFWEVISDEHGIDPSGNYVGDSDLQLERISVYYNEASSHKYV   60
                             ******************:**************:*.***** ******.*****:*::***

F7CB79 F7CB79_MONDO     61   PRAILVDLEPGTMDSVRSGPFGQLFRPDNFIFGQTGAGNNWAKGHYTEGAELVDSVLDVV  120
Q13509 TBB3_HUMAN       61   PRAILVDLEPGTMDSVRSGAFGHLFRPDNFIFGQSGAGNNWAKGHYTEGAELVDSVLDVV  120
                             ******************* **:***********:************************

F7CB79 F7CB79_MONDO     121  RKECEHCDCLQGFQLTHSLGGGTGSGMGTLLISKIREEYPDRIMNTFSVMPSPKVSDTVV  180
Q13509 TBB3_HUMAN       121  RKECENCDCLQGFQLTHSLGGGTGSGMGTLLISKVREEYPDRIMNTFSVVPSPKVSDTVV  180
                             *****:*****************************:**************:**********

F7CB79 F7CB79_MONDO     181  EPYNATLSVHQLVENTDETYCIDNEALYDICFRTLKLTTPTYGDLNHLVSATMSGVTTSL  240
Q13509 TBB3_HUMAN       181  EPYNATLSIHQLVENTDETYCIDNEALYDICFRTLKLATPTYGDLNHLVSATMSGVTTSL  240
                             ********:*****************************:**********************

F7CB79 F7CB79_MONDO     241  RFPGQLNADLRKLAVNMVPFPRLHFFMPGFAPLTARGSQQYRALTVPELTQQMFDAKNMM  300
Q13509 TBB3_HUMAN       241  RFPGQLNADLRKLAVNMVPFPRLHFFMPGFAPLTARGSQQYRALTVPELTQQMFDAKNMM  300
                             ************************************************************

F7CB79 F7CB79_MONDO     301  AACDPRHGRYLTVATVFRGPMSMKEVDEQMLAIQNKNSSYFVEWIPNNVKVAVCDIPPRG  360
Q13509 TBB3_HUMAN       301  AACDPRHGRYLTVATVFRGRMSMKEVDEQMLAIQSKNSSYFVEWIPNNVKVAVCDIPPRG  360
                             ******************* ***************.************************

F7CB79 F7CB79_MONDO     361  LKMASTFIGNSTAIQELFKRISEQFSAMFRRKAFLHWFTGEGMDEMEFTEAESNMNDLVS  420
Q13509 TBB3_HUMAN       361  LKMSSTFIGNSTAIQELFKRISEQFTAMFRRKAFLHWYTGEGMDEMEFTEAESNMNDLVS  420
                             ***:********************:***********:**********************

F7CB79 F7CB79_MONDO     421  EYQQYQEATANDGEETFEDDEEEMNE----                              446
Q13509 TBB3_HUMAN       421  EYQQYQDATAEEEGEMYEDDEEESEAQGPK                              450
                             ******:***::  * :****** :
```

Identical positions	415
Identity	92.222%
Similar positions	23
Program	CLUSTALO

Figure 8. Alignment of opossum and human tubulin beta chain protein sequence. Comparison between opossum and relevant human protein sequence (highlighted). The protein of interest is tubulin beta chain, encoded by TUBB6 (opossum) or TUBB3 (human). There are 415 identical positions in sequences, and identity of 92.22%. Based on sequence similarity, reactivity of these antibodies with opossum cannot be predicted with certainty.

In order to test if the antibodies react specifically with opossum, we immunostained cortical cell cultures from P17 after 19 days *in vitro*. Visualization showed that all antibodies react with Tubulin III of opossum, but with different quality of staining. We observed that rabbit polyclonal anti-ß-Tubulin III (Fig. 9, A) and TUJ1 (Fig. 9, B) work well on opossum cortical cultures. Unlike SOX2 and SOX9 which are nuclear markers, these antibodies have cytoplasmic localization and they label neurons. On the other hand, monoclonal anti-ß-Tubulin III IgG2a (Fig. 9, C) does not seem to be specific enough - it stains other cells in addition to neurons. Finally, monoclonal anti-ß-Tubulin III IgG2b (Fig. 9, D) works poorly on the opossum cortex. Labeling is unclear and therefore this antibody is not suitable for the staining of neurons in our cell cultures.

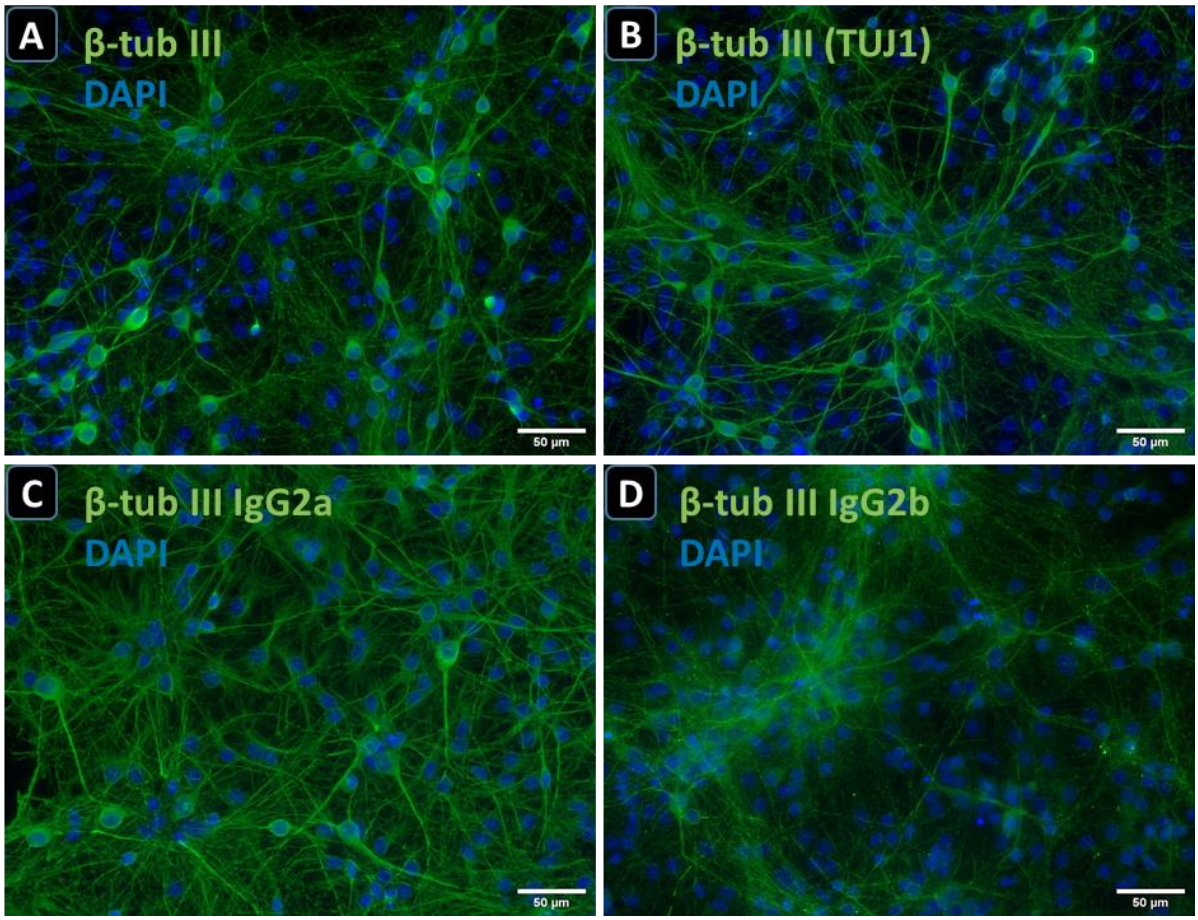


Figure 9. Validation of four different anti-β-tubulin III primary antibodies on opossum cortical primary cell cultures from P17 at DIV19. The cortical primary cells from P17 opossum were fixed at DIV19. Cell cultures were labeled with four different anti-ß-Tubulin III (green). **A** Rabbit polyclonal (Catalog # T2200). **B** Mouse (TUJ1) monoclonal IgG2a (Catalog #801201). **C** Mouse monoclonal IgG2a (Catalog #66240-1-Ig). **D** Mouse monoclonal IgG2b (Catalog #T5076). Cellular nuclei were stained for DAPI (blue). Data are obtained from single experiment. Magnification 20x, scale bars, 50 µm.

We further examined the specificity of monoclonal anti-ß-Tubulin III IgG2a. By immunostaining spinal cord cultures from P17 at DIV7 with anti-ß-Tubulin III IgG2a and SOX9 (Fig. 10, A) we confirmed that anti-ß-Tubulin III IgG2a stains all present cells (neurons, fibroblasts, glial cells), which means that is not neuron-specific as it should be. However, in Fig. 10 we can observe that neurons can be distinguished from other cells by their morphology. Importantly, neurons are not SOX9+ (Fig. 10, B). Therefore, we can conclude that ß-Tubulin III and SOX9 are mutually exclusive neuronal and glial markers.

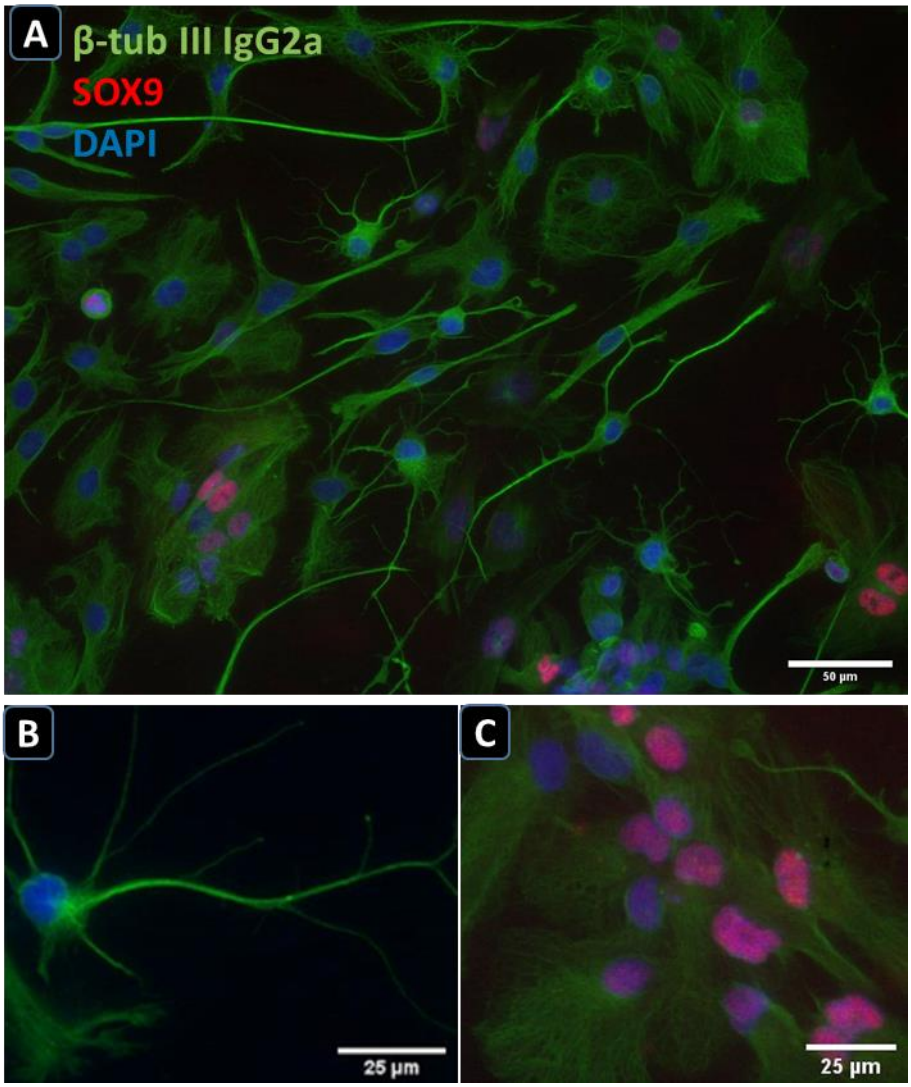


Figure 10. ß-Tubulin III and SOX9 are mutually exclusive. The spinal cord primary cells from P17 opossum were fixed at DIV7. **A** Cell cultures were labeled with monoclonal anti-ß-Tubulin III IgG2a (green) and SOX9 (red). All present cells are positive on ß-Tubulin III IgG2a, which means that the antibody is not specific for neurons. **B** Neurons do not express SOX9, they are TUJ1+/SOX9-. **C** SOX9 is expressed by other cells present in culture (probably glial precursor cells). Cellular nuclei were stained for DAPI (blue). Two experiments were performed. Magnification 20x, scale bars **A** 50 µm; **B, C** 25 µm.

After testing different ß-Tubulin III antibodies, we confirmed that TUJ1 is a neuron-specific marker that works excellently on opossum. TUJ1 was chosen for further experiments with primary cortical and spinal cord cultures. In order to examine the time-dependent changes that occur in cell morphology, as well as changes in the expression of the markers, cultures were fixed at two different time points, DIV1 and DIV7. Cell cultures were immunostained following the ICC protocol (previously described in Materials and Methods). To label cell nuclei, we used DAPI. Images were taken at 20x magnification with a fluorescence microscope. For quantification, we manually counted DAPI+, TUJ1+, SOX2+, and SOX9+ cells. Additionally, we also counted double-positive cells, TUJ1+/SOX2+ as well as SOX2+/SOX9+. All quantifications were performed using the ImageJ plugin Cell Counter. Afterwards, we calculated the proportion of every marker to DAPI in order to obtain the percent of cell types present in P5 and P16 cell cultures (Fig. 11).

4.3.2 Immunostaining of cortical primary cell cultures

Cortical primary cell cultures deriving from neonatal (P5) opossum express high levels of ß-Tubulin III (TUJ1+ cells). Because TUJ1 is a marker for immature neurons, we observe that there are ~47% neuronal cells in the culture at DIV1, and at DIV7 the percentage is even higher, around 64% (Fig. 12, A). SOX2, a NSC marker, shows high expression at DIV1. Moreover, there is a significant decrease in its expression during culturing (Fig. 12, B). It is important to note that all cultures were maintained in neuronal medium with 5% FBS. Co-expression of TUJ1 and SOX2 remained unchanged during cultivation, at around ~1% (Fig. 12, C). Next, the expression of SOX9+/SOX2- is low and with no significant difference between DIV1 and DIV7 (Fig. 12, D). Cells that co-express SOX2 and SOX9 are present in the culture in ~24% at DIV1, but expression decreases during cultivation (Fig. 12, E).

Cell cultures deriving from older (P16) opossums have an unchanged expression of TUJ1 during cultivation. About 30% of all cells (DAPI positive) are TUJ1+ neurons (Fig. 12, A). The expression of SOX2+/TUJ1-/SOX9- decreases at DIV7 in comparison to DIV1 (Fig. 12, B). Co-expression of TUJ1 and SOX2 is highest in P16 at DIV1, around ~2% (Fig. 12, C white arrow). A decrease in expression at DIV7 is also observed in TUJ1+/SOX2+ (Fig. 12, C) and SOX9+/SOX2- (Fig. 12, D) cells. The only significant increase during cultivation is observed in the co-expression of SOX2 and SOX9. At DIV1 the SOX2+/SOX9+ cells represent ~15%, while at DIV7 their ratio increases up to ~40% (Fig. 12, E white arrow).

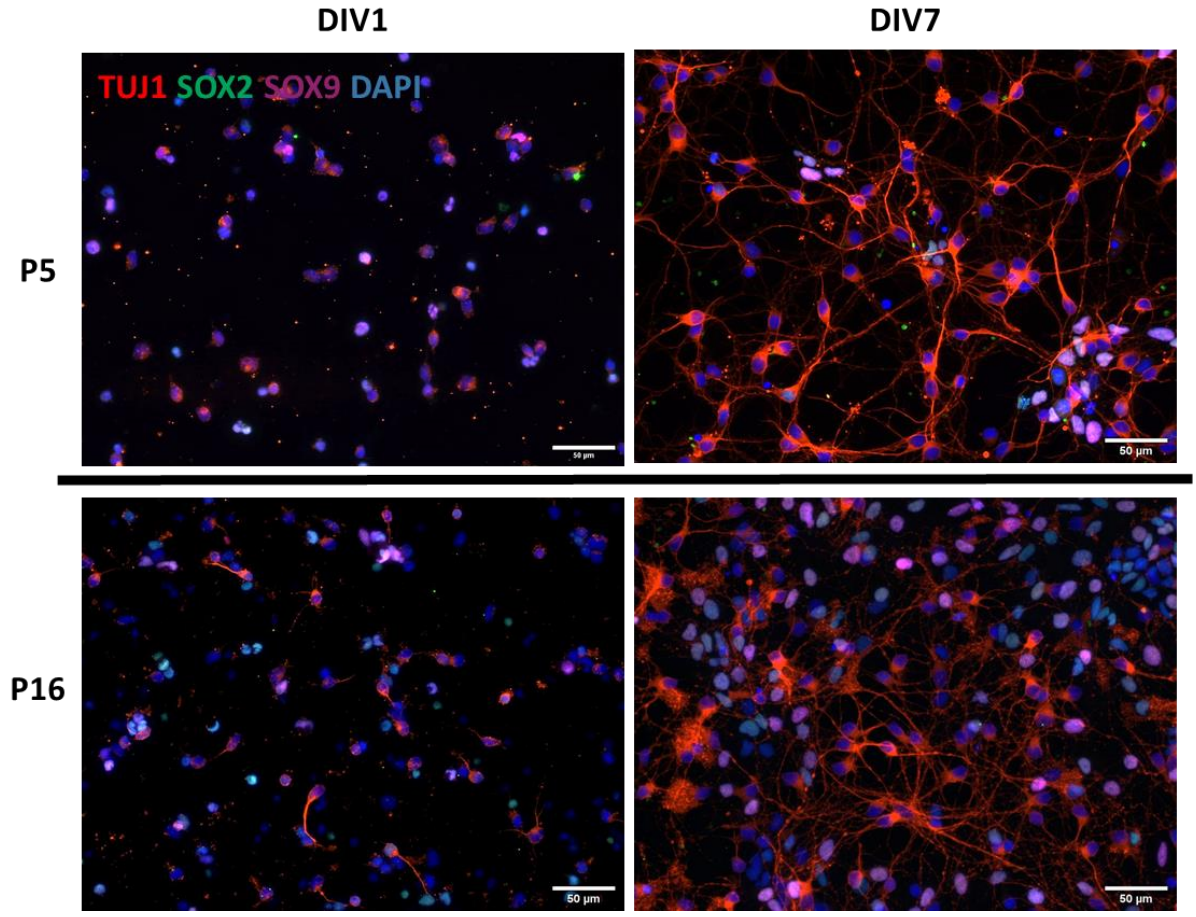
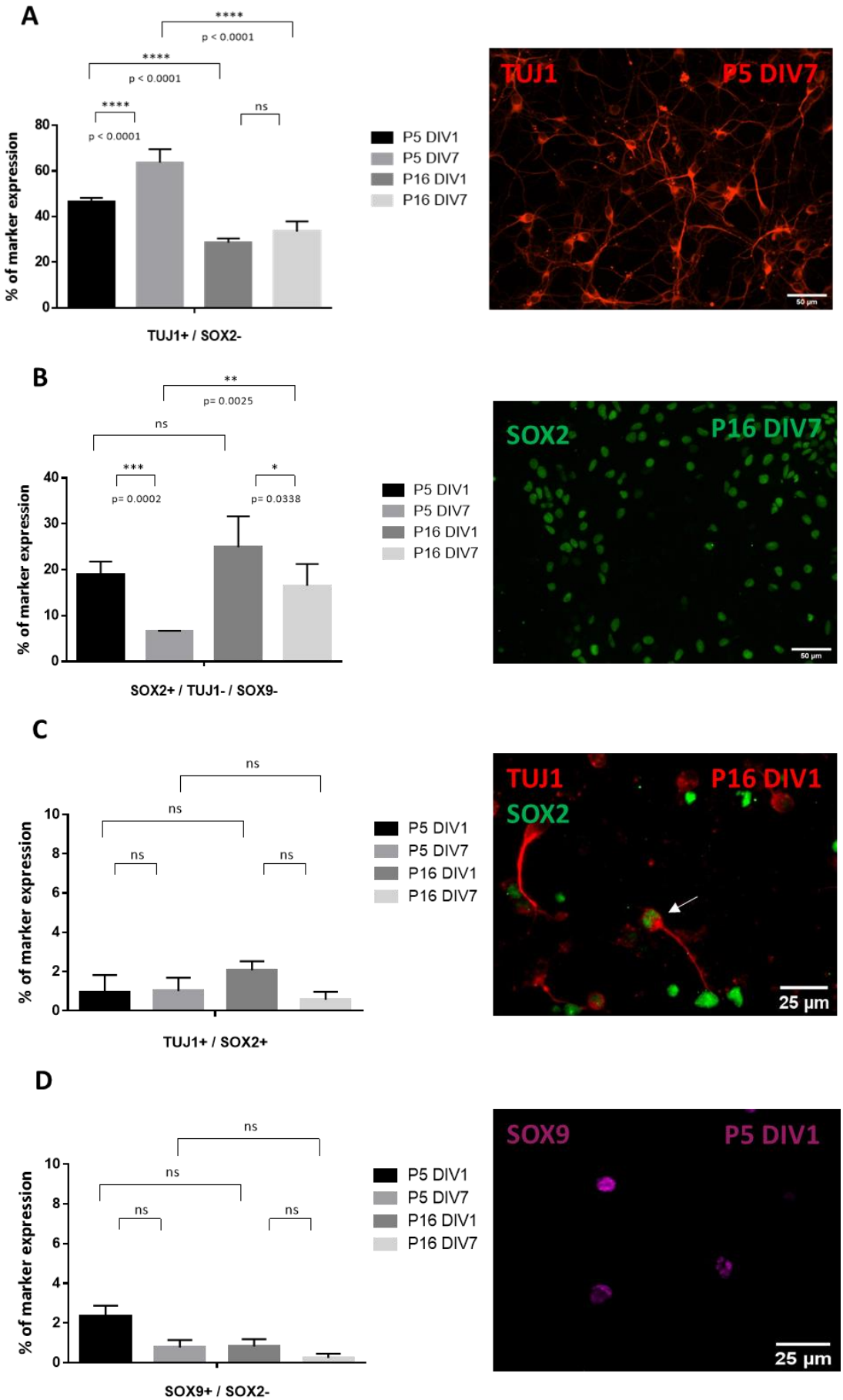


Figure 11. Fluorescent images of cortical primary cell cultures from P5 and P16 opossum at two different stages during culture, DIV 1 and DIV7.

The cortical primary cells from P5 (upper row) and P16 (bottom row) opossum were fixed with 4% PFA, permeabilized with 0.1% Triton X-100 in PBS, and stained at DIV1 and DIV7. Cell cultures were triple labeled with anti-TUJ1 (red), anti-SOX2 (green) and anti-SOX9 (magenta) antibody. Cellular nuclei were stained for DAPI (blue). Colors have been adjusted using ImageJ. Three experiments were performed (n=3 cultivated cortical cell cultures). Magnification 20x, scale bars, 50 µm.


A
****
p < 0.0001
****
p < 0.0001
****
p < 0.0001
ns
% of marker expression
TUJ1+ / SOX2-
P5 DIV1
P5 DIV7
P16 DIV1
P16 DIV7
TUJ1
P5 DIV7
50 µm
B
**
p= 0.0025
ns
***
p= 0.0002
*
p= 0.0338
% of marker expression
SOX2+ / TUJ1- / SOX9-
P5 DIV1
P5 DIV7
P16 DIV1
P16 DIV7
SOX2
P16 DIV7
50 µm
C
ns
ns
ns
ns
% of marker expression
TUJ1+ / SOX2+
P5 DIV1
P5 DIV7
P16 DIV1
P16 DIV7
TUJ1
SOX2
P16 DIV1
25 µm
D
ns
ns
ns
ns
% of marker expression
SOX9+ / SOX2-
P5 DIV1
P5 DIV7
P16 DIV1
P16 DIV7
SOX9
P5 DIV1
25 µm

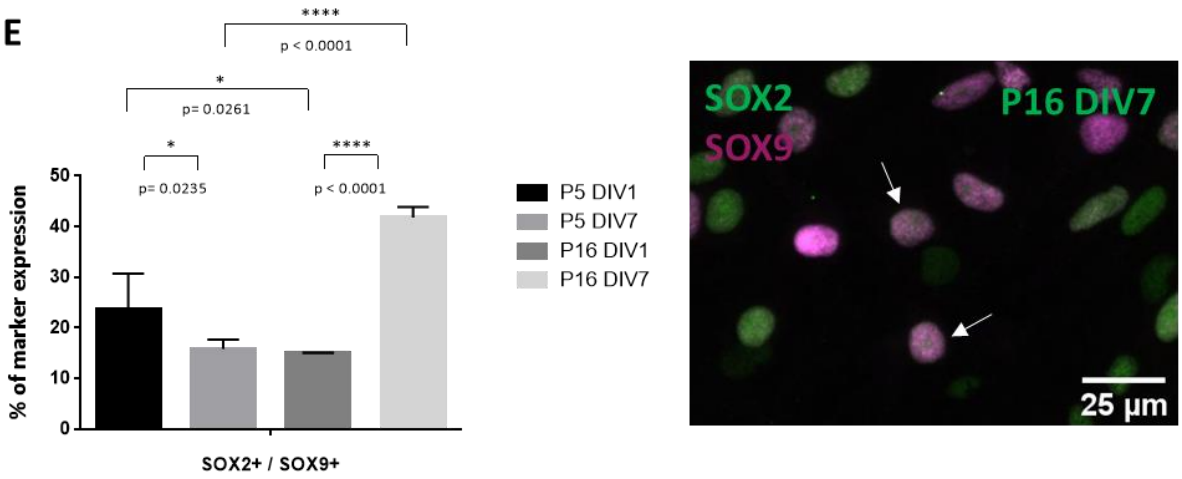
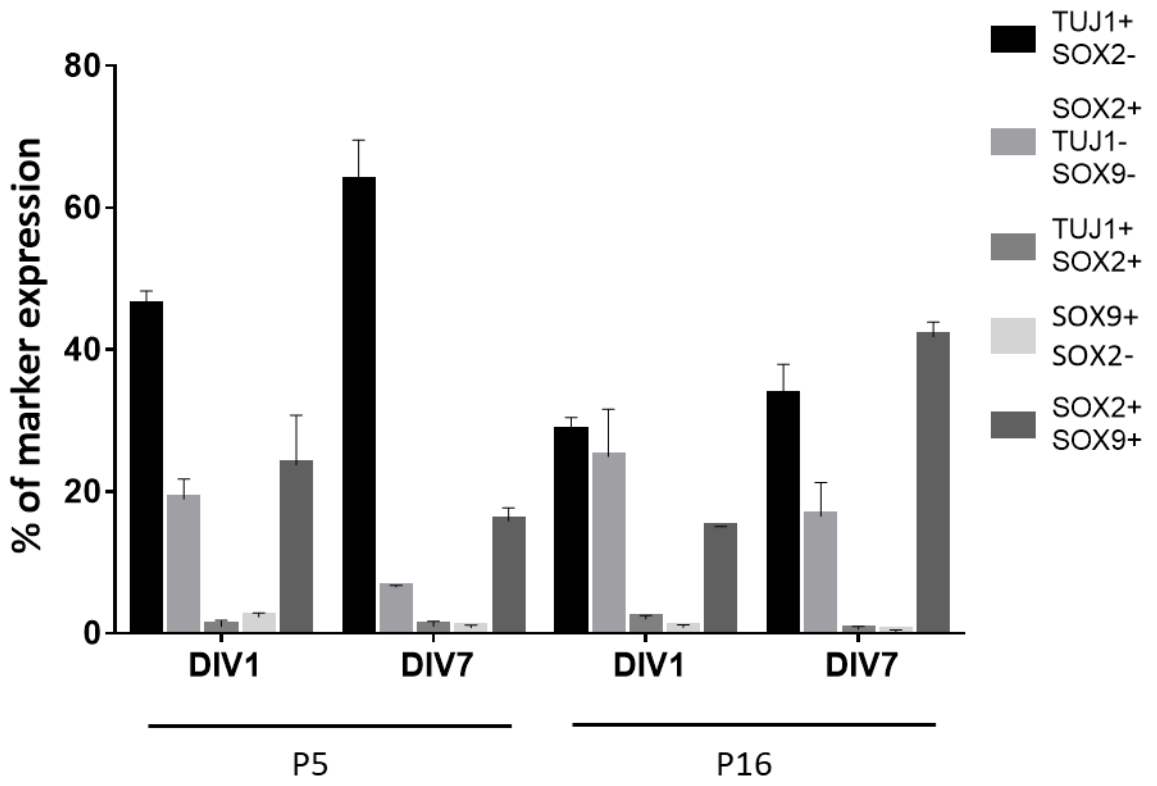


Figure 12. Changes in marker expression in the DIV1 and DIV7 cortical cell cultures deriving from P5 and P16 opossums.

Bar graph represents percentage of TUJ1+/SOX2- expression (**A**, red), SOX2+/TUJ1-/SOX9- (**B**, green), co-expression of TUJ1 and SOX2 (**C**, red; green) SOX9+/SOX2- (**D**, magenta) and co-expression of SOX2 and SOX9 (**E**, green; magenta) in P5 and P16, in two different time points, DIV1 and DIV7. On the right of each, graph there is a corresponding fluorescent image of stained cell culture in which marker of interest is highly expressed. White arrows point towards cells that co-express TUJ1+/SOX2+ (**C**) and SOX2+/SOX9+ (**E**). Labeled cells were quantified using the ImageJ plugin Cell Counter. Data are shown as mean ± SD of three experiments [two-way ANOVA, $p<0.05$, $n=3$ cultivated cortical cell cultures, 5 images were counted per each experiment]. Colors have been adjusted using ImageJ. Magnification 20x, scale bars **A, B** 50 µm; **C, D, E** 25 µm.

By combining all the obtained data from P5 and P16 at DIV1 and DIV7 (Fig. 13) we concluded that P5 cultures have a high percentage of neuronal cells labeled with TUJ1, and that there is a significant decrease in expression between DIV7 from P5 and P16. Also, the percentage of neuronal cells in P16 remains unchanged throughout the cultivation period (Fig. 12, A). The expression of SOX2 has the same pattern both in P5 and P16 cultures. There is no significant difference in expression at DIV1, but there is a significant decrease during the cultivation (Fig. 12, B). Cells that co-express TUJ1 and SOX2 are most abundant in P16 cultures at DIV1 (Fig. 12, C). In general, expression of SOX9+/SOX2- is very low, but has its peak at DIV1 of P5 (Fig. 12, D). Finally, in P16 cultures there is a significant increase of cells expressing SOX2 and SOX9 (Fig. 12, E).

A



B

	P5 DIV1	P5 DIV7	P16 DIV1	P16 DIV7
TUJ1+ / SOX2-	46.23%	63.71%	28.55%	33.50%
SOX2+/ TUJ1-/ SOX9-	18.93%	6.54%	24.91%	16.50%
TUJ1+ / SOX2+	0.93%	1.00%	2.06%	0.56%
SOX9+ / SOX2-	2.34%	0.77%	0.82%	0.23%
SOX2+ / SOX9+	23.74%	15.85%	15.03%	41.83%

Figure 13. Graphical and tabular display of marker expression in the DIV1 and DIV7 cortical cell cultures deriving from P5 and P16 opossums.

A Bar graph shows combined data obtained by analysis of time-dependent immunoprofiling cortical primary cell cultures. Changes in markers were observed at P5 (left) and P16 (right) animals at DIV1 and DIV7. In P5 cultures expression of TUJ1+/SOX2- is significantly higher than in P16. In both cultures, there is a significant decrease in the expression of SOX2+/TUJ1-/SOX9- during cultivation. There is no significant difference in the expression of SOX9+/SOX2- neither co-expression TUJ1+/SOX2+. In P16 cultures SOX2+/SOX9+ cells are in abundance, especially at DIV7. **B** Table represents numeric mean percentage for each marker at DIV1 and DIV7 in P5 and P16 cortical cell cultures. Labeled cells were quantified using the ImageJ plugin Cell Counter. Data are shown as mean ± SD of three experiments [two-way ANOVA, $p<0.05$, n=3 cultivated cortical cell cultures, 5 images were counted per each experiment]. Colors have been adjusted using ImageJ.

4.3.3 Immunostaining of spinal cord primary cell cultures

In the spinal cord primary cell cultures derived from younger opossums, there is a significant increase in TUJ1 expression during cultivation (Fig. 14). At DIV1, about 40% of total cells represent neuronal cells, and at DIV7 that percentage reaches around 65% (Fig. 15, A). There is no significant increase in expression of SOX2+/TUJ1-/SOX9- (Fig. 15, B), TUJ1+/SOX2+ co-expression (Fig. 15, C) nor SOX9+/SOX2- (Fig. 15, D) during cultivation. Finally, co-expression of SOX2 and SOX9 is present in ~20% of cells at DIV1, but their expression slightly decreases during cultivation (Fig. 15, E).

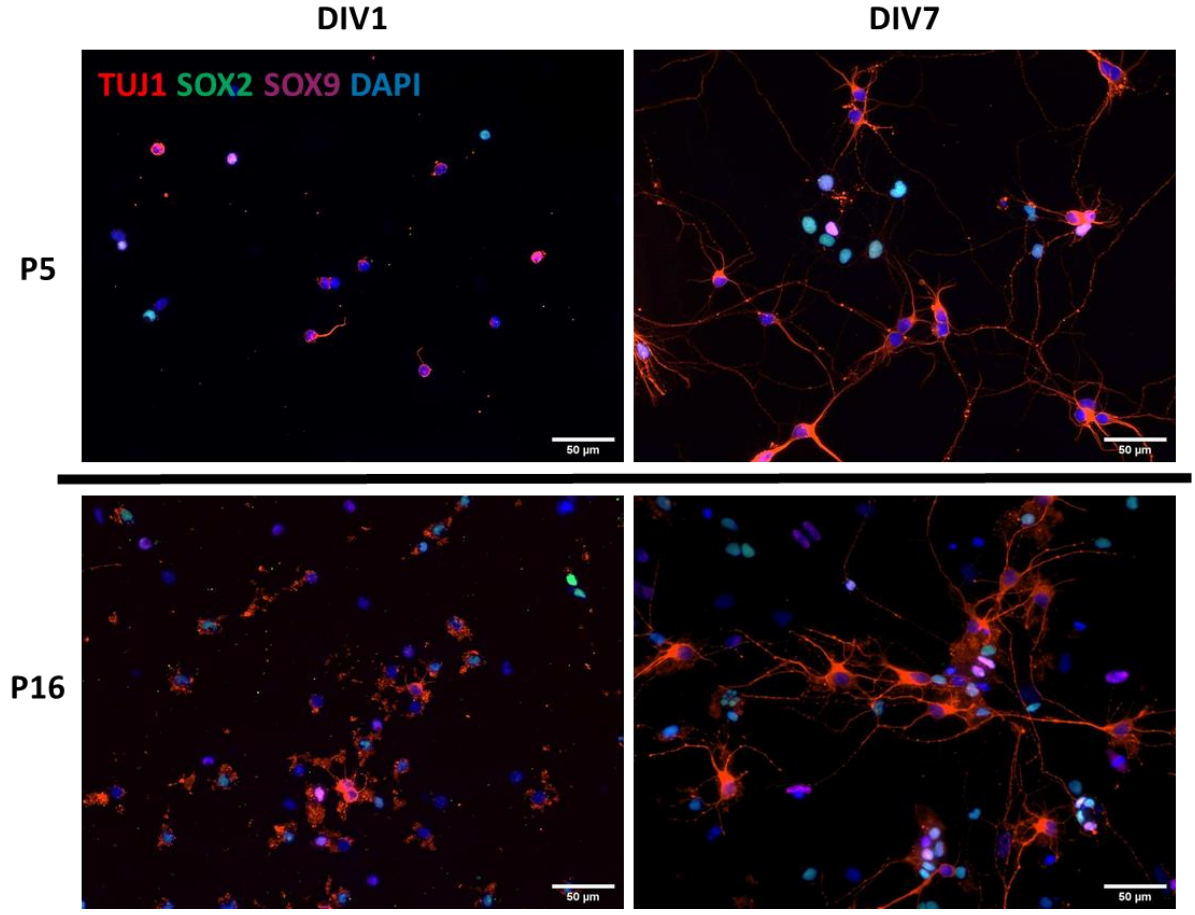
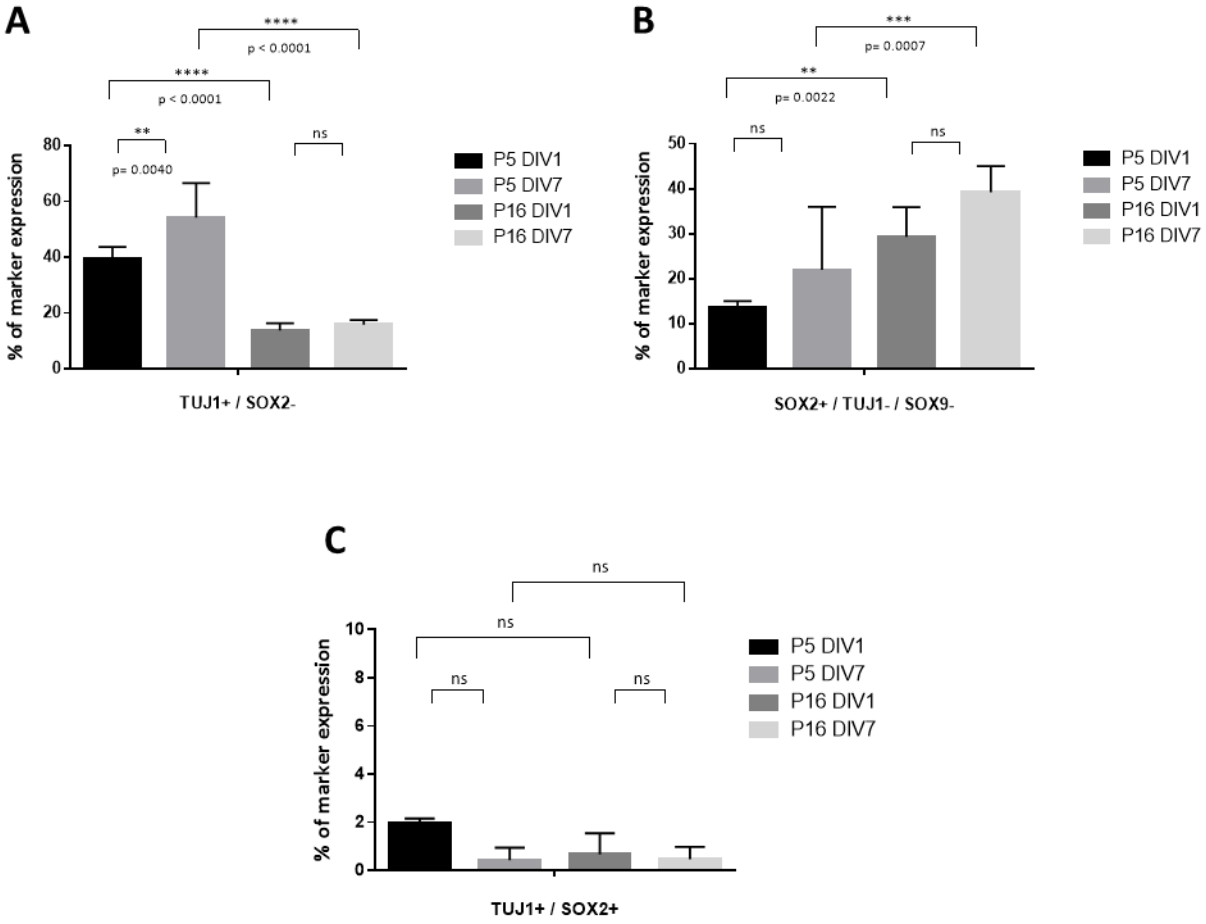


Figure 14. Fluorescent images of spinal cord primary cell cultures from P5 and P16 opossum at two different stages during culture, DIV 1 and DIV7.

The spinal cord primary cells from P5 (upper row) and P16 (bottom row) opossum were fixed with 4% PFA, permeabilized with 0.1% Triton X-100 in PBS, and stained at DIV1 and DIV7. Cell cultures were triple labeled with anti-TUJ1 (red), anti-SOX2 (green) and anti-SOX9 (magenta) antibody. Cellular nuclei were stained for DAPI (blue). Colors have been adjusted using ImageJ. Data are obtained from three experiments (n=3 cultivated spinal cord cell cultures). Magnification 20x, scale bars, 50 µm.

Cell cultures derived from older opossums have a slight increase in expression of TUJ1 during cultivation. Only about 15% of DAPI positive cells are TUJ1+, which is two times less than in cortical cell cultures (Fig. 15, A). The expression of SOX2+/TUJ1-/SOX9- increases at DIV7 in comparison to DIV1 (Fig. 15, B). As in P5 cultures, expression of TUJ1+/SOX2+ (Fig. 15, C) and SOX9+/SOX2- (Fig. 15, D) remains unchanged during cultivation. There is no significant difference in the co-expression of SOX2 and SOX9, only a small decrease at DIV7. At DIV1 ~20% of cells are SOX2+/SOX9+, while at DIV7 their percentage slightly decreases to ~17% (Fig. 15, E).



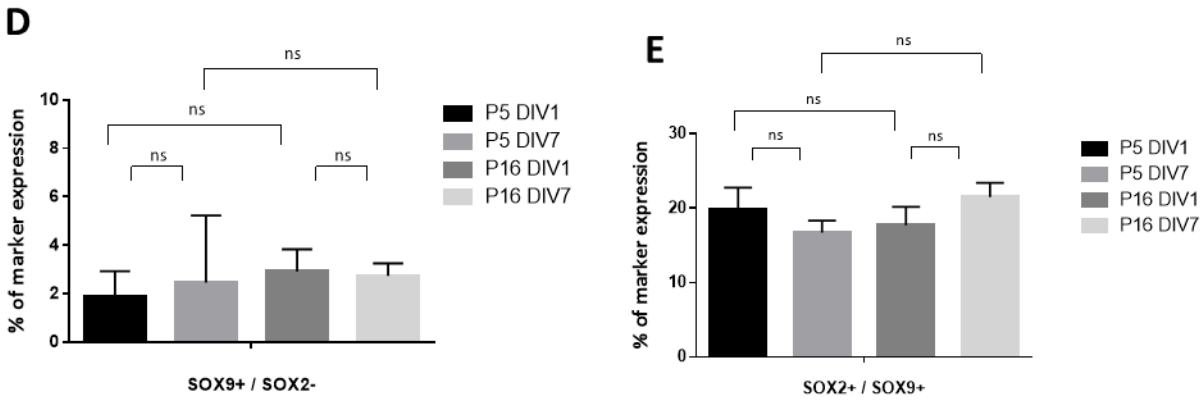
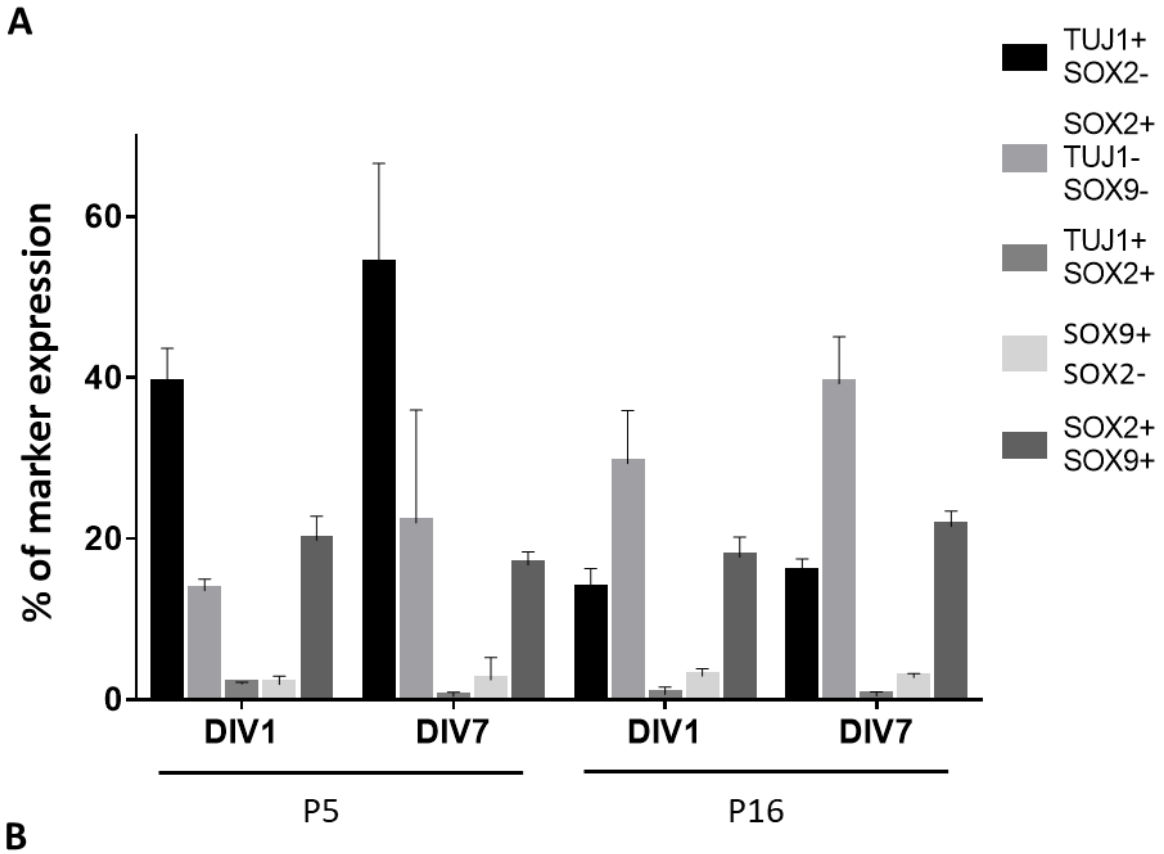


Figure 15. Changes in marker expression in the DIV1 and DIV7 spinal cord cell cultures deriving from P5 and P16 opossums.

Bar graph represents the percentage of TUJ1+/SOX2- expression (**A**), SOX2+/TUJ1-/SOX9- (**B**), co-expression of TUJ1 and SOX2 (**C**) SOX9+/SOX2- (**D**) and co-expression of SOX2 and SOX9 (**E**) in P5 and P16, in two different time points, DIV1 and DIV7. Labeled cells were quantified using the ImageJ plugin Cell Counter. Data are shown as mean ± SD of three experiments [two-way ANOVA, $p<0.05$, $n=3$ cultivated spinal cord cell cultures, 5 images were counted per each experiment].

Taken together, our data (Fig. 16) show that P5 cultures have a significantly higher percentage of neuronal cells (labeled with TUJ1 in both DIV1 and DIV7) than P16 cultures. Moreover, in P16 the percentage of TUJ1+ remains unchanged during cultivation (Fig. 15, A). The expression pattern of SOX2+/TUJ1-/SOX9- remains the same for both P5 and P16 cultures. There is a slight increase in the expression of SOX2+/TUJ1-/SOX9- during the cultivation period (Fig. 15, B). The percentage of TUJ1 and SOX2 co-expression is low, mostly present in P5 cultures at DIV1 (Fig. 15, C). Overall, the expression of SOX9+/SOX2- remains unchanged during cultivation (Fig. 15, D). Finally, there is no significant difference in the co-expression of SOX2 and SOX9, only a small increase at DIV7. At DIV1 ~18% of cells are double-positive for SOX2+ and SOX9+, and ~22% at DIV7 (Fig. 15, E).

A



B

	P5 DIV1	P5 DIV7	P16 DIV1	P16 DIV7
TUJ1+ / SOX2-	39.19%	54.15%	13.79%	15.28%
SOX2+/ TUJ1-/ SOX9-	13.55%	21.96%	29.31%	39.23%
TUJ1+ / SOX2+	1.97%	0.43%	0.67%	0.47%
SOX9+ / SOX2-	1.91%	2.46%	2.93%	2.74%
SOX2+ / SOX9+	19.78%	16.73%	17.22%	21.51%

Figure 16. Graphical and tabular display of marker expression in the DIV1 and DIV7 spinal cord cell cultures deriving from P5 and P16 opossums.

A Bar graph shows combined data obtained by analysis of time-dependent immunoprofiling spinal cord primary cell cultures. Changes in markers were observed at P5 (left) and P16 (right) animals at DIV1 and DIV7. In P5 cultures expression of TUJ1+/SOX2+ is significantly higher than in P16. The expression of SOX2+/TUJ1-/SOX9- is increasing during cultivation in both cultures. Also, there is a significant difference between SOX2+/TUJ1-/SOX9- expression at DIV1 from P5 and P16. Expression of SOX9+/SOX2- as well as the co-expression TUJ1+/SOX2+, remains low during cultivation. In co-expression of SOX2 and SOX9, there is no significant difference in expression. **B** Table represents numeric mean percentage for each marker at DIV1 and DIV7 in P5 and P16 spinal cord cell cultures. Labeled cells were quantified using the ImageJ plugin Cell Counter. Data are shown as mean ± SD of three experiments [two-way ANOVA, $p<0.05$, n=3 cultivated cortical cell cultures, 5 images were counted per each experiment]. Colors have been adjusted using ImageJ.

In comparison to primary cultures from the cortex (Fig. 13, A) , both P5 and P16 spinal cord cultures (Fig. 16, A) have less neuronal cells. However, the increase in the expression of certain markers during cultivation is common. For instance, while in cortical cultures expression of SOX2+/TUJ1-/SOX9- decreases during the cultivation, in spinal cord cultures expression increases from DIV1 to DIV7, for both ages. Moreover, the percentage at DIV7 from P16 spinal cord is twice as high in comparison to DIV7 from P16 cortex. The co-expression of TUJ1 and SOX2 is slightly higher in cortical cultures, while expression of SOX9+/SOX2- is higher in the spinal cord. Finally, co-expression of SOX2 and SOX9 is also higher in cortical cell cultures, especially at P16 DIV7.

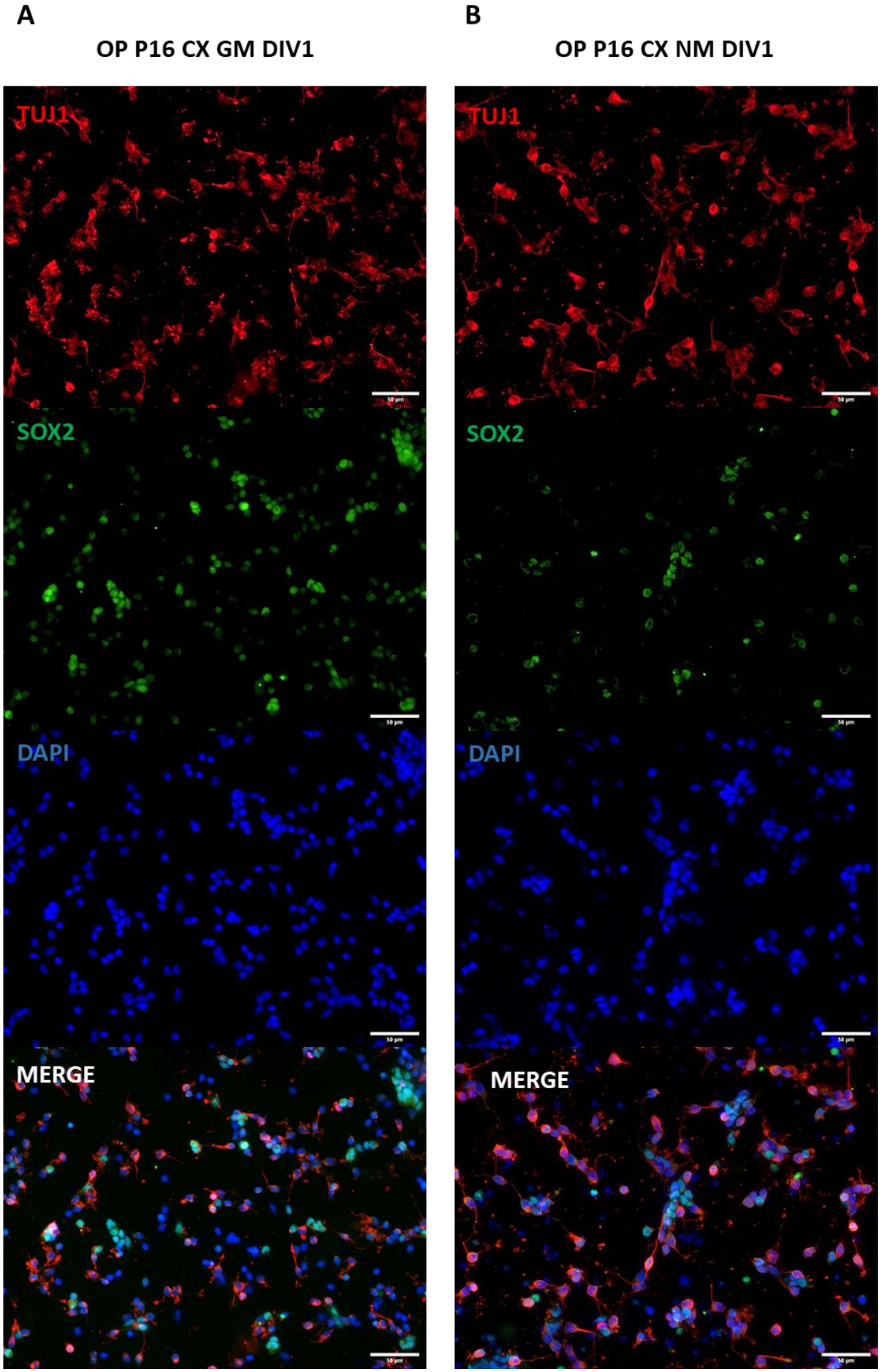
4.4 Primary neuronal cultures in different plating medium

In order to examine the impact of the medium composition on cell growth and differentiation, we tested two different plating mediums. We checked whether the FBS-rich glial medium (GM), with a higher concentration of growth factors, will promote proliferation and expansion of the NSCs and whether neuronal medium (NM) will favor neuronal differentiation.

We decided to conduct this experiment with P16 cortical cell cultures because these cultures have a higher yield of cells than spinal cord cultures (compare Fig. 6 and Fig. 7). The cell cultures were maintained in the incubator for 1 DIV, and then ICC was performed. This time point was chosen to verify which cell types survive after dissection and to analyze neurite outgrowth. The primary antibodies that were used were TUJ1 and anti-SOX2 (Fig. 17, A, B).

Cell cultures cultivated in GM (Fig. 17, A) have a higher percentage of SOX2+/TUJ1- cells, as well as double-positive TUJ1+/SOX2+ cells (Fig. 17, E). In Fig. 17, C the white circle indicates a neuron that co-expresses TUJ1 and SOX2. There is a significant decrease in the percentage of cells expressing SOX2+ alone and TUJ1+/SOX2+ in co-expression in NM (Fig.17, E). The white arrow in Fig. 17, D points to a neuron that is TUJ1+/SOX2-. The expression of TUJ1+/SOX2- cells is approximately equal both in GM and NM, which indicates that the medium composition does not affect the neuron number (Fig. 17, E).

However, cultures maintained in NM (Fig. 17, B) show a significant difference in the length of neurites. In comparison to the GM (Fig. 17, A), neurites in NM are visibly longer, which confirms that NM promotes neuronal differentiation and neurite outgrowth. We conclude that GM indeed favors the proliferation of SOX2+ neural stem/progenitor cells, as well as TUJ1+/SOX2+ cells, while NM promotes neuronal differentiation.


A
OP P16 CX GM DIV1
TUJ1
SOX2
DAPI
MERGE
B
OP P16 CX NM DIV1
TUJ1
SOX2
DAPI
MERGE

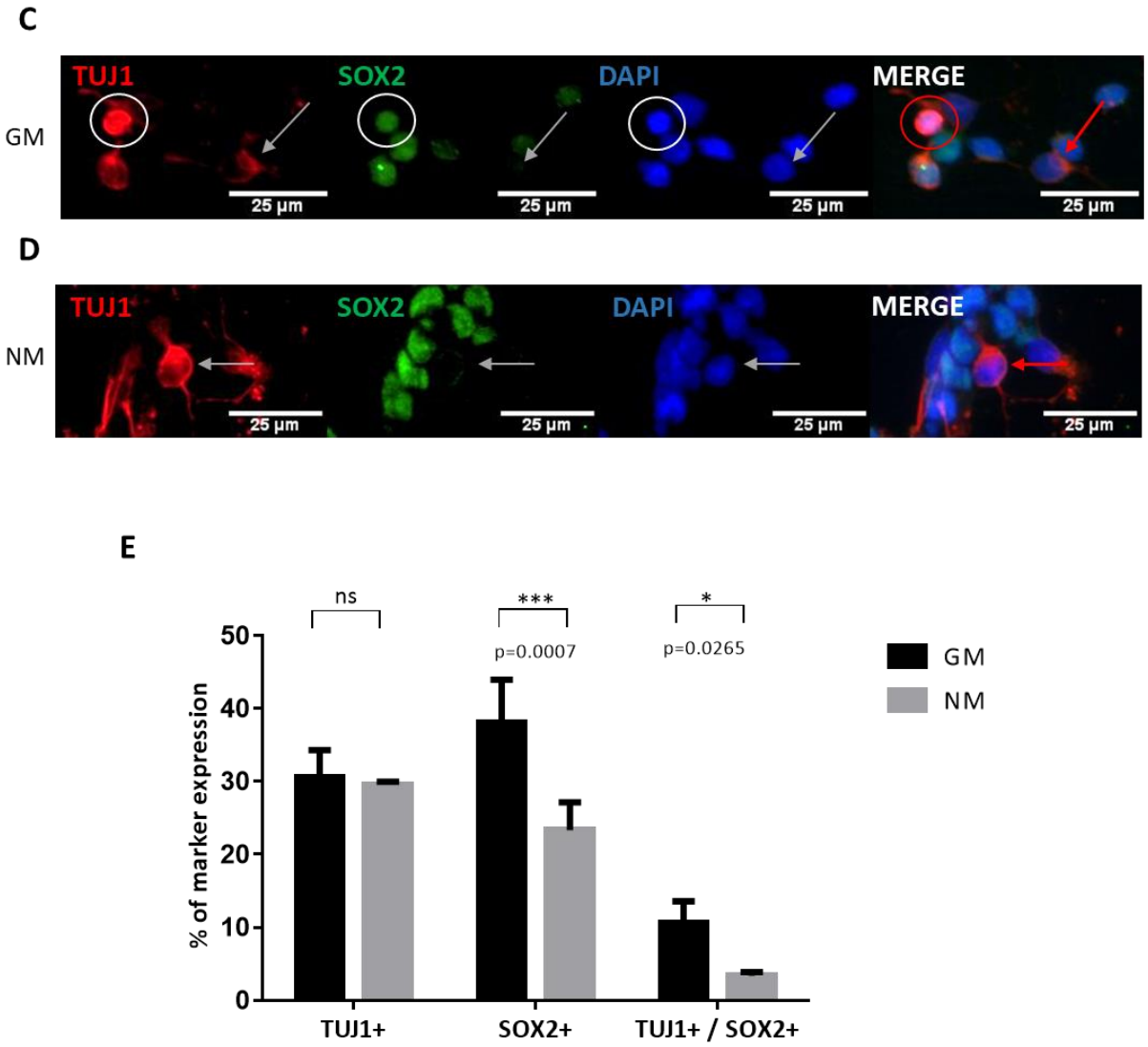


Figure 17. Glial medium favors proliferation and abundance of neural stem/progenitor cells while neuronal medium promotes neuronal differentiation.

A, B show fluorescence microscopy images of P16 cortical primary cell cultures in different plating media. **A** Culture maintained in GM for proliferation; **B** Culture maintained in NM for differentiation. The cells were cultivated 1 DIV, fixed with 4% PFA and immunostained with neuronal (TUJ1, red) and stem/progenitor (SOX2, green) marker at RT. Cellular nuclei were stained for DAPI (blue). Magnification 20x; scale bars, 50 µm. **C** White circle identifies neuron from GM that is TUJ1+/SOX2+, and white arrow points toward neuron that is only TUJ1+; **D** White arrow identify neuron from NM that is TUJ1+/SOX2-. Scale bar, 25 µm. Color and contrast have been adjusted using the ImageJ. **E** Bar graph shows the percentage of TUJ1+ and SOX2+ markers in GM and NM. Labeled cells were quantified using the ImageJ plugin Cell Counter. Data represent mean ± SD from three independent experiments (two-way ANOVA, $p<0.05$). There is a significant decrease in the percentage of cells expressing SOX2+/TUJ1- and cells co-expressing TUJ1 and SOX2 in NM in comparison to GM.

4.5 Characterization of opossum fresh-fixed spinal cord tissue using immunohistochemistry

In dissociated primary cultures, the original tissue 3D architecture is lost. Therefore, to characterize and compare cell populations in our cultures with spinal cord intact tissue, we performed IHC on spinal cords of opossums of different ages, P5 and P16. The tissue sections were double-labeled with anti-SOX2, anti-SOX9 and cell nuclei were stained with DAPI. As shown in Fig. 18, images of three different segments of the P5 and P16 spinal cord (central canal, ventral and dorsal column) were obtained with fluorescence microscope. For quantification, we counted cells labeled with DAPI and SOX2+ or SOX9+ cells, and then double-labeled cells (both SOX2 and SOX9 positive). All quantifications were performed using the ImageJ plugin Cell Counter.

Immunofluorescence staining of lumbar spinal cord sections from P5 (Fig. 19) shows that more than 40% of DAPI positive cells are co-expressing SOX2 and SOX9. These double-positive cells are highly expressed around the central canal (CC; Fig. 19, middle column). Also, 5% of SOX2+/SOX9+ cells are localized in the ventral column (VC; Fig. 19, right column), while in the dorsal column (DC; Fig. 19, left column) are less than 1%. SOX2+/SOX9- cells are in ~20% present around CC (Fig. 19) and in a small percentage in the VC (~8%, Fig. 19). In the DC there is only 1% of SOX2+/SOX9-. We observed that SOX9+/SOX2- is in ~3% present in the CC, and less than 1% in the VC and DC (Fig. 19).

In the lumbar spinal cord sections from P16, there is a decrease in expression of SOX2+/SOX9+ around the CC (Fig. 20). Furthermore, on fluorescence images it is visible that most cells are scattered around the CC, except that SOX2+/SOX9- cells localized along one end of the canal. However, in other segments of the spinal cord, there is an increase in expression of SOX2+/SOX9+, thus in ventral part ~26% of SOX2+/SOX9+ is localized, and in dorsal ~8% (Fig. 20, right and left column). There is an

increase in expression of SOX2+/SOX9- cells in every segment of the spinal cord. About 26% of the SOX2+/SOX9- cells are present around the CC, 23% in VC and 10% in DC. Finally, there is a decrease in SOX9+/SOX2- cells around the CC, but a small increase in both the ventral and dorsal segments of the spinal cord (Fig. 20).

We concluded that expression of cells that co-express SOX2 and SOX9 is highest in P5 around the CC and then, during development, that occurs a decrease which is observed in P16 (Fig. 21, A). Interestingly, there is an obvious difference in the localization of SOX2+/SOX9+ cells. In P5 cells that co-express high levels of SOX2 and SOX9 are mostly located narrowly along the CC (Fig. 19), while in P16 cells are scattered around (Fig. 20). In P5 ventral and dorsal segments there are only a few marker positive cells, while in P16 there are many SOX2+/SOX9+ (Fig. 21, D) and SOX2+/SOX9- cells (Fig. 21, B). Finally, expression of SOX9+/SOX2- (Fig. 21, C) is generally very low, but there is a slight increase observed in P16.

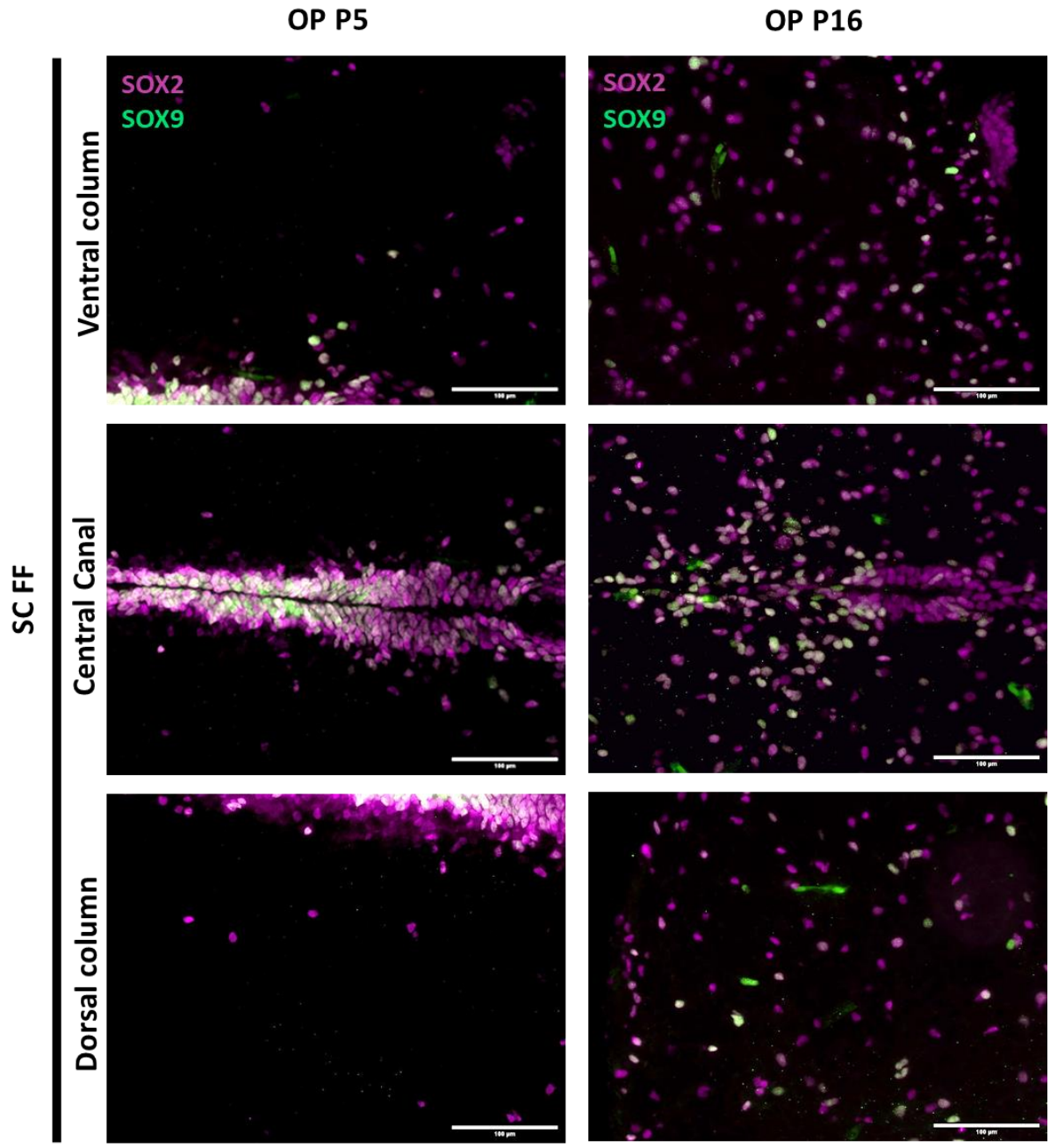
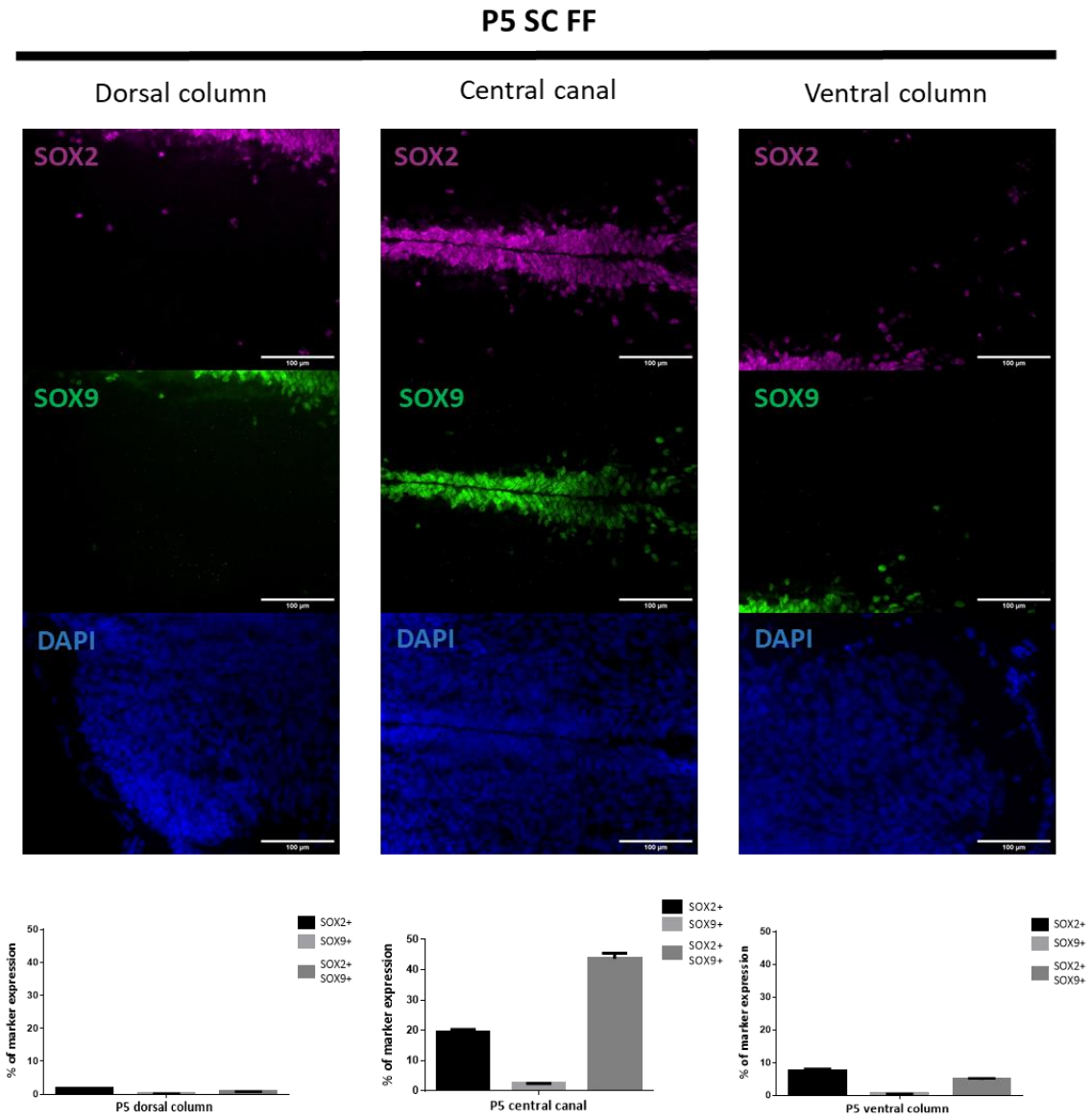


Figure 18. Distribution and localization of SOX2 and SOX9 in the fresh-fixed opossum spinal cord that can (P5, left column) and cannot (P16, right column) regenerate.

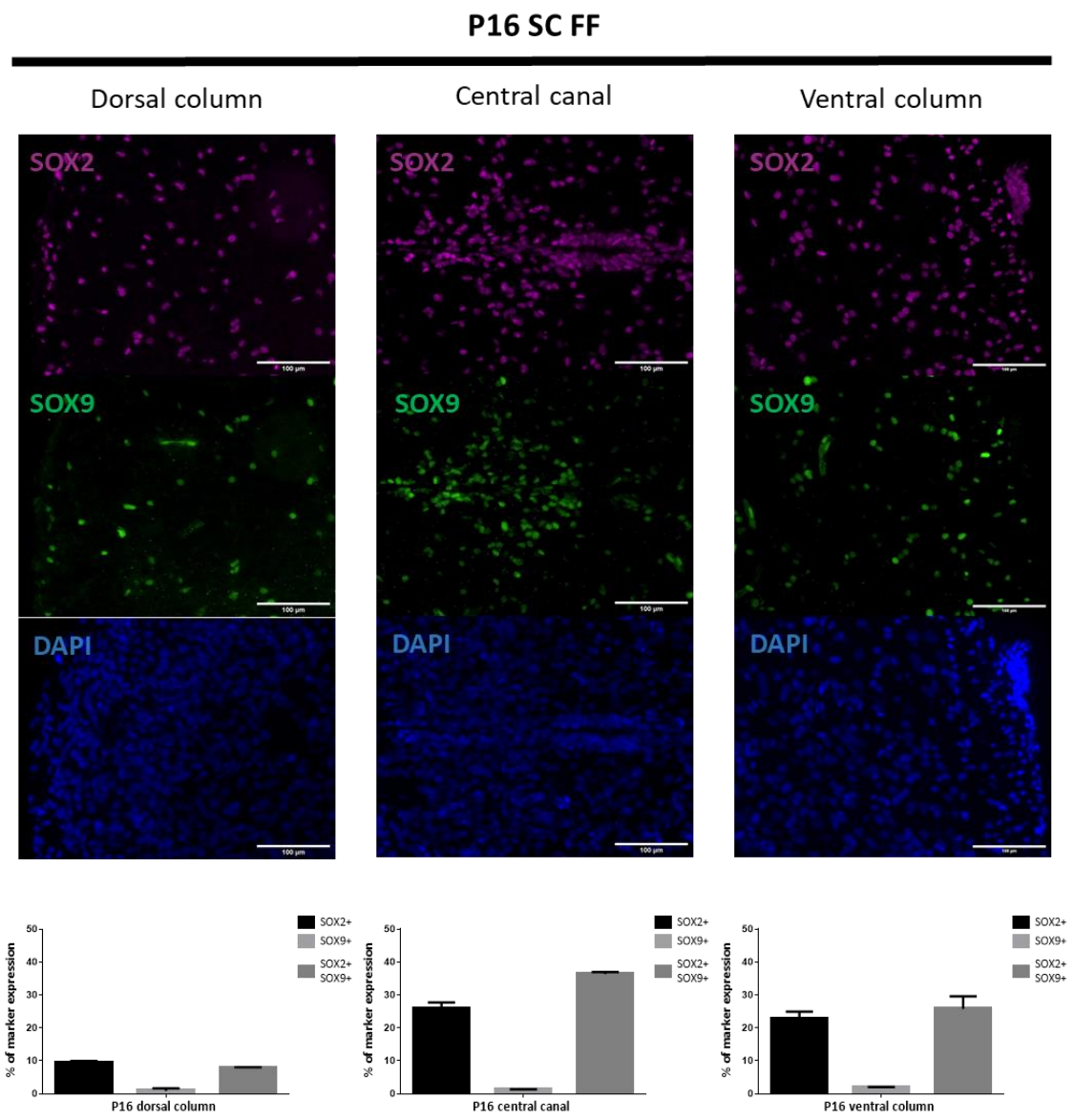
The spinal cord tissue from animals of different age was immunostained with stem/progenitor (SOX2, magenta) and stem/glial progenitor marker (SOX9, green) at RT. This figure shows fluorescence microscope images of three different segments of P5 spinal cord: VC (top row), CC (middle row) and DC (bottom row). Two canals are merged together to present distribution and localization of SOX2 and SOX9 in P5 and P16 SC FF. Remarkable differences are observed in every segment of younger and older spinal cord. In VC and DC there is higher expression of SOX2+/SOX9- and SOX9+/SOX2- in P16 than it is in P5. In P5 co-expression of SOX2 and SOX9 is present along the CC, while in P16 those cells are scattered around. In P16 there is also expression of SOX2+/SOX9- narrowly along the one end of the CC. Magnification 20x; scale bars, 100 μm. Abbreviations: DC, dorsal column; CC, central canal; VC, ventral column; SC, spinal cord; FF, fresh-fixed.



SOX2+ SOX9-	1.64%	19.11%	7.29%
SOX9+ SOX2-	0.11%	2.30%	0.32%
SOX2+ SOX9+	0.78%	43.69%	4.70%

Figure 19. SOX2+/SOX9+ and SOX2+/SOX9- are highly expressed around the CC in the P5 fresh-fixed spinal cord.

Fluorescence microscope images of three different segments of P5 SC: DC (left), CC (middle) and VC (right). The tissue preparations were immunostained with anti-SOX2 (magenta) and anti-SOX9 (green) at RT. Below images there are corresponding bar graphs that show percentage of SOX2+/SOX9-, SOX9+/SOX2- and SOX2+/SOX9+ cells in each segment individually. Data represent mean ± SD obtained from two experiments [two-way ANOVA, $p<0.05$, $n=2$ opossum spinal cords for each age group]. The range on the Y-axis is manually determined in order to represent the values correctly. In addition, there is a table with numeric mean values for each marker below graphs. Labeled cells were quantified using the ImageJ plugin Cell Counter. Magnification 20x; scale bars, 100 μm.



SOX2+ SOX9-	9.30%	25.80%	22.56%
SOX9+ SOX2-	0.90%	1.27%	1.82%
SOX2+ SOX9+	7.69%	36.38%	25.79%

Figure 20. SOX2+/SOX9+ are highly expressed around the CC and in the VC in the P16 fresh-fixed spinal cord.

Fluorescence microscope images of DC (left), CC (middle) and VC (right) of P16 SC. For IHC were used primary antibodies anti-SOX2 (magenta) and anti-SOX9 (green). Below images there are corresponding bar graphs that show percentage of SOX2+/SOX9-, SOX9+/SOX2- and SOX2+/SOX9+ cells in each segment individually. Data represent mean ± SD obtained from two experiments [two-way ANOVA, $p<0.05$, $n=2$ opossum spinal cords for each age group]. The range on the Y-axis is manually determined in order to represent the values correctly. In addition, there is a table with numeric mean values for each marker below graphs. Labeled cells were quantified using the ImageJ plugin Cell Counter. Magnification 20x; scale bars, 100 μm.

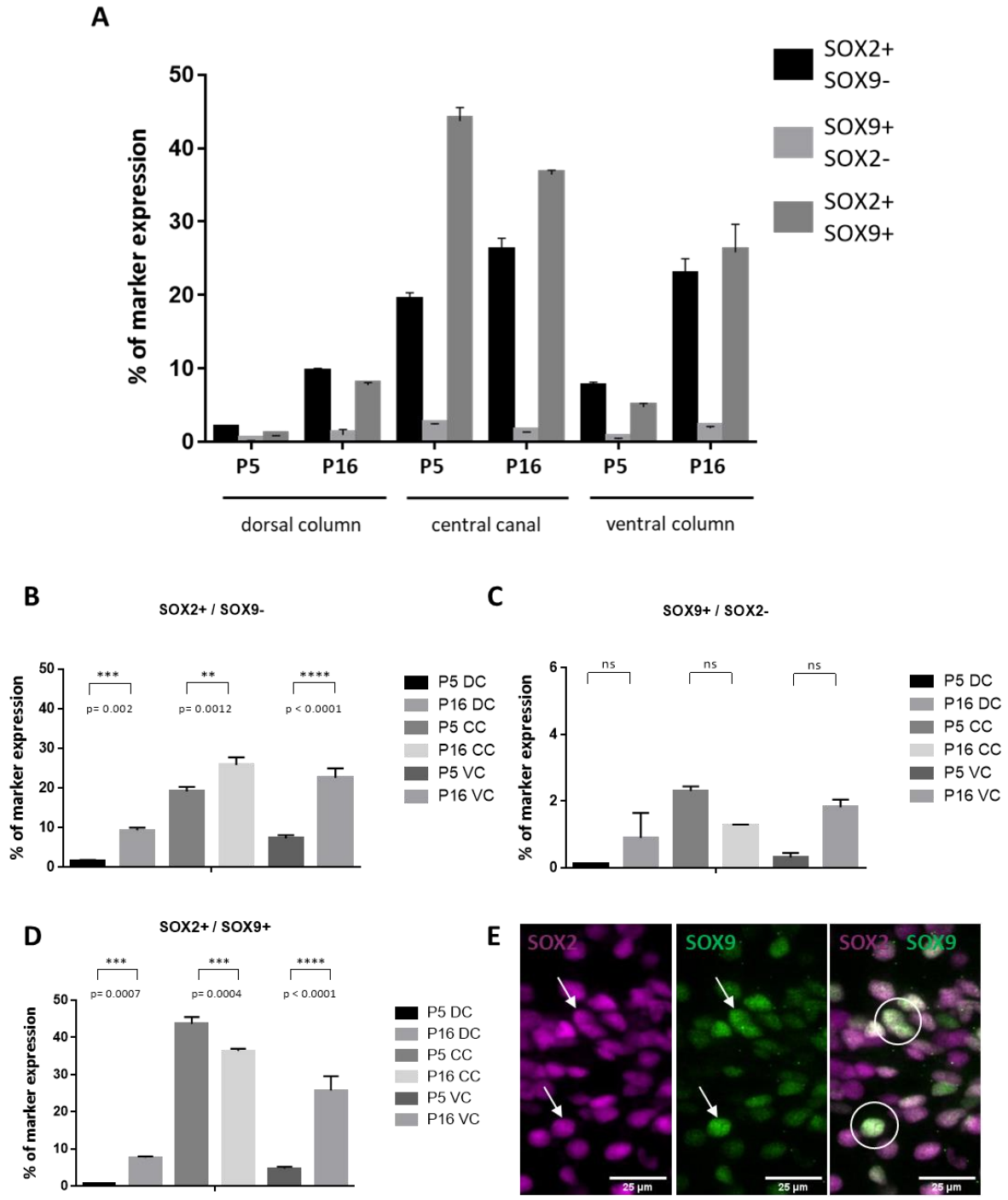


Figure 21. Cells that co-express SOX2 and SOX9 are the most abundant cells in P5 and P16 fresh-fixed spinal cord.

A Bar graph shows combined results obtained by analysis of immunostained tissue preparations. Changes in marker expression between DC, CC and VC of P5 and P16 spinal cord are shown. **B** Bar graph represents percentage of SOX2+/SOX9- expression in DC, CC and VC of P5 and P16 spinal cord. **C** Bar graph represents percentage of SOX9+/SOX2- expression in DC, CC and VC of P5 and P16 spinal cord. **D** Bar graph represents percentage of cells co-expressing SOX2 and SOX9 in DC, CC and VC of P5 and P16 spinal cord. **A, B, C, D** Data are shown as mean ± SD of two experiments [two-way ANOVA, $p<0.05$, $n=2$ opossum spinal cords for each age group, 3 images counted per different segment]. **E** White circle identify cells that express both SOX2 and SOX9. Magnification 20x; scale bars, 25 µm.

5. Discussion

The ability to prepare *in vitro* primary neuronal cultures has been essential for making advances in the, still poorly understood, molecular basis and functioning of the CNS. Several studies have demonstrated the usefulness of isolated CNS of neonatal opossum as an *in vitro* preparation (23), but dissociated primary neuronal cultures from opossums have not yet been established. Here we present data for both cortical and spinal cord primary cell cultures from neonatal opossum that were established for the first time. In this thesis, cortical (Fig. 6) and spinal cord (Fig. 7) primary cultures were prepared from opossums of different age, P5 and P16. The survival and growth of the cultures was observed through time-dependent changes in cell morphology at two critical time-points, DIV1 and DIV7.

At DIV1, in both cortical and spinal cord cultures, the existence of a certain amount of debris is observed. As stated in similar protocols (47), debris is due to the remains of myelin from spinal cord tissue or partially removed meninges. During development, the meninges are more adherent to the tissue and because of that removing them during dissection becomes a major challenge (28). To provide a cleaner environment for cells, it is important to remove the debris by changing half of the medium at DIV1.

Despite the debris, cell survival and neurite outgrowth was observed in both cortical and spinal cord. The highest survival rate and neural density is present in cortical P16 cultures at DIV7 (Fig. 6). This could be due to the larger size of the P16 brain compared to P5, which therefore contains more neurons. Seelke et al. (18) reported that neuronal density in the opossum neocortex is highest around P18, which corresponds to our results. However, in P16 spinal cord cultures at DIV7 (Fig. 7), we observed that a majority of cells do not have neuron-like morphology. Indeed, confirming the fact that neurogenesis is followed by gliogenesis (48), our results show that later stages of development are correlated with a higher proportion of glial cells in P16 spinal cord cultures.

Another observation refers to the yield of cells that was significantly different, between cortical and spinal cord cultures. In comparison to cortical cultures (Fig. 6), spinal cord cell cultures (Fig. 7) have visibly lower yield of cells both at DIV1 and DIV7. Although the exact reason behind it is hard to determine, there are few possibilities. Firstly, for effective dissociation of the robust spinal cord tissue (connective tissue in particular), a stronger enzymatic treatment was performed, which could influence the survival of the cells (47). Secondly, it is harder to remove the meninges from the spinal cord than those from the brain, especially in older opossums when the meninges are sticky. Therefore, it is possible to lose a part of spinal cord tissue while removing meninges, which ultimately results in cell culture with a smaller yield. Finally, we should not exclude the possibility that spinal cord neurons have a lower survival rate in culture than cortical neurons. Indeed, we confirm that the preparation of spinal cord cultures is more challenging in comparison to cortical cultures.

Here, primary cell cultures were prepared and maintained in neuronal medium with 5% FBS in order to characterize and compare their cellular content using ICC. In particular, we examined the expression of stem cell markers SOX2 and SOX9. Neuronal differentiation was detected by staining with ß-Tubulin III. Prior to performing ICC, we tested if those antibodies react specifically with an opossum. Validation of different primary antibodies for ß-Tubulin III on opossum cortical cultures (Fig. 9) confirmed that TUJ1 is a neuron-specific marker (49) suitable for the staining of immature neurons, without staining glia.

However, during this experiment, we came across another important result. In comparison to TUJ1, the monoclonal β-tubulin III IgG2a antibody showed cross-reactivity with glial cells in spinal cord cultures. As a result, all cells in our culture were positive for β-tubulin III, including SOX9+ cells (Fig. 10, A). Previously conducted studies showed that SOX9 drives the differentiation towards gliogenesis, indicating that the expression of SOX9 in NSCs continues in glial cells, but not in neurons (39). Moreover, another

study reported that no co-expression of SOX9 and NeuN was observed in the immunostained mouse cortex (42). Based on these data, expression of ß-Tubulin III and SOX9 is mutually exclusive, thus we defined co-expression as a false-positive staining. This co-expression can be explained by the fact that glial cells in opossum express an isotype of β-tubulin that antibodies for class III do not recognize in humans. However, there are differences between opossum TUBB6 and human TUBB3 proteins. Because sequences share 92.22% of identity (Fig. 8), it is very likely that part of a sequence that antibody recognizes in human is different in the opossum. We can conclude that neurons, both immature and mature, do not express SOX9 (Fig. 10, B).

After preparing the cultures and validating antibodies, ICC was performed. During development, different cell types undergo dramatic changes in their size and function. Newborn, post-mitotic neurons express TUJ1, and our data show that neurons represent predominant cells in P5 cortical cell cultures (Fig. 11). The ratio of neurons is highest in younger opossums and then decreases at P16 (Fig. 12, A), which corresponds to the previous investigations. According to Seelke et al. (18), in the opossum neocortex neuronal density is highest during neurogenesis, at P18. Although the number of neurons remains unchanged through the lifespan, the percentage of neurons decreases with maturation, which we have confirmed. Next, SOX2+/TUJ1-/SOX9- (Fig. 12, B) cells were identified as stem/progenitor cells that can differentiate both in neurons and glia (32). We observed that during cultivation at P5 and P16 the percentage of neurons increases, while the percentage of stem/progenitor cells decreases. This result implies that SOX2+ cells differentiate into neurons. In previous research it was shown that most SOX2+ cells are exclusive of TUJ1+, although some SOX2+/TUJ1+ were found in the VZ and the SVZ. Based on marker positivity and location, these cells can be identified as IPCs (40) or it indicates that downregulation of SOX2 is gradual in opossum neurons. Finally, SOX2+/SOX9+ cells significantly increase at P16 during

cultivation (Fig. 12, E). We identified these cells as stem/glial progenitor cells. Seelke et al. (18) showed that around P18 there is an onset of gliogenesis followed by a remarkable increase in the number of non-neuronal cells. Based on the expression of markers, these cells could be identified as NECs or RGCs. However, we cannot identify these cells with certainty because both populations express SOX2 as well as SOX9. Moreover, the exact time point of switching NECs to RGCs is still unknown and debatable (36). We concluded that through the development of the opossum cortex there is an increase both in the size and in the total number of cells. However, the percentage of neurons generally decreases, while the percentage of non-neuronal cells increases (Fig. 13, A).

Much less is known about the spinal cord development in opossums. Our results (Fig. 16) show that the percentage of neurons significantly increases during the early developmental stages, and then decreases with maturation. This data is equivalent to the data from cortical cell culture (Fig. 13). Furthermore, during development, the percentage of stem/progenitor cells in spinal cord tissue increases. The presence of SOX2 inhibits premature neuronal differentiation and promotes proliferation (40). If we compare the expression of TUJ1 and SOX2 in spinal cord cultures, we can observe that the expression of TUJ1 is decreased when SOX2 expression is increased. By inhibiting neuronal differentiation, SOX2 maintains cells in the neural precursor state. In spinal cord culture, there is only a small percentage of IPCs, as well as SOX9+.

Together, data obtained by ICC of both cortical and spinal cord cell cultures suggest that in both tissues neurons represent predominant cells, which confirms that neuronal density is highest during cortical neurogenesis, at P18 (18). Our results show that the ratio of neurons is higher in cortical cultures than spinal cord. Furthermore, we confirmed the presence of stem/progenitor cells that express SOX2+. According to various studies (40), levels of SOX2 have an important role in the specification of NPCs. Moreover, high expression of SOX2 leads to inhibition of neurogenesis and

promotes gliogenesis. Our results show that expression of TUJ1+/SOX2- and TUJ1-/SOX2+ is inversely proportional, meaning that an increase in SOX2 leads to a decrease in TUJ1, and *vice versa*. According to Hutton and Pevny (40), TUJ1+/SOX2+ can be found in the VZ and the SVZ. In our cultures, the ratio of IPCs is higher in cortical cultures (Fig. 12, C), because the VZ and the SVZ are situated in the brain tissue. Finally, the stem/glial progenitor SOX2+/SOX9+ cells cannot be identified based on the expression on these two markers. However, we can for sure conclude that SOX2+/SOX9+ are lineage-restricted glial precursors. SOX9 was previously described as a NSC component involved in switching from neurogenesis to gliogenesis (43). Regarding their expression in cultures, there is a significant increase in cortical P16 cultures at DIV7 (Fig. 12, E), approximately time point that corresponds to the onset of gliogenesis.

Furthermore, in this study, we tested two different plating mediums to observe if the medium composition affects cell growth and differentiation. ICC performed on P16 cortical cultures at DIV1 has shown that cultures cultivated in GM are rich in stem/progenitor cells, while NM promotes neurite outgrowth and differentiation (Fig. 17). Moreover, in cultures cultivated in GM, there is a higher ratio of TUJ1+/SOX2+ cells, which are identified as IPCs (40). The number of neurons is the same in both cultures. Thus, we can conclude that the composition of the culture medium is an important factor and with different medium, we can manipulate our cell cultures. The knowledge obtained enables us to develop protocols that will aim to maintain the abundance of certain cells in culture.

Finally, IHC was performed on transverse sections of wild type spinal cord from P5 (Fig. 19) and P16 (Fig. 20) opossums in order to compare results with data obtained by ICC. In addition to the percentage of marker expression, using IHC we also examined the distribution and localization of markers in the preserved tissue architecture that is lost in primary cultures. Results show that cells co-expressing SOX2+/SOX9+ are the largest population both in P5 and P16 lumbar spinal cord. However, there is a

significant difference in their localization that occurred during development. While in P5 cells are located narrowly along the CC, in P16 they are scattered around and present in the VC and DC as well. By combining the results of different studies that examined SOX2 and SOX9 expression in the developing spinal cord, we identified SOX2+/SOX9+ cells as stem/glial progenitor cells. There is a possibility that these cells are NECs, earliest progenitors in the CNS that are situated around CC and express SOX2 (Fig. 3). Furthermore, Farrell et al. (50) showed that SOX9 is also expressed very early in NECs and remains expressed in late differentiating glial cells. However, because the exact time point of switching NECs to RGCs is still unknown and debatable, we cannot identify these cells as NECs with certainty. It is important to highlight that these cells can also be identified as RGCs because RGCs express both SOX2 (40) and SOX9 (43). To identify this cell population, further experiments need to be performed. For instance, we could examine the expression of astroglial markers that distinguish RGCs from NECs, like PAX6 (38).

Other than SOX2+/SOX9+ cells, in P16 (Fig. 20) there is a significant ratio of SOX2+/SOX9- cells. Specifically, these cells are located at the one end of CC in P16, after most of the SOX2+/SOX9+ cells have migrated to the VC or DC. According to Hugnot and Franzen (33), in adult rodent spinal cord, NSCs are situated in the ependymal region, which corresponds to CC. Moreover, most of the ependymal cells express SOX2 and show properties of NSCs. Finally, the expression of SOX9+/SOX2- is unremarkable which is in accordance with result that SOX9 expression is present only in the ventricular zone (CC) and absent in the roof and floor plates of the developing spinal cord (50).

Although we are not able to identify cells in P5 around CC with certainty, we can conclude that those are stem/glial progenitor cells. During development, these cells proliferate, differentiate and migrate to the other parts of spinal cord leaving only SOX2+ NSCs narrowly along the CC. We assume that these cells are NSCs that will persist into adulthood.

6. Conclusion

In this thesis, we prepared opossum cortex and spinal cord primary cultures derived from animals of different age and different regenerative capacity. Cortex and spinal cord cultures were characterized and compared using immunofluorescence at two different time points, DIV 1 and 7. Markers of interest were SOX2, SOX9, and TUJ1. We tested different cell culture media to develop an optimal protocol for the maintenance of either the stem/progenitor cells or neurons in the culture. Furthermore, we characterized the expression and localization of mentioned markers in opossum fresh-fixed spinal cord tissue preparations. The following was determined:

1. the expression of TUJ1, neuron-specific marker and stem/glial progenitor SOX9 is mutually exclusive
2. neurons are predominant cells at P5 cortical as well as spinal cord cell cultures
3. at P16 cultures the presence of SOX2 expression correlates with neuronal differentiation and stem/glial progenitor cells proliferation
4. GM favors proliferation and expansion of the neural stem cells while NM promotes neuronal differentiation
5. stem/progenitor cells that co-express SOX2 and SOX9 in P5 spinal cord are around CC, while in P16 they are scattered
6. NSCs are present in P16 narrowly along the CC

Considering the fact that cortex and spinal cord primary cell cultures from neonatal opossum were established for the first time, this will require further characterizations. Firstly, it is important to continue with the characterization of cortical and spinal cord cell cultures. To identify cell type with certainty, we need to examine the expression of different markers. Future studies will be based on using primary cell cultures for genetic manipulation of the activity of candidate molecules that may have an important role in promoting or preventing regeneration.

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16/07/2018–Present

Associate researcher

Laboratory for Molecular Neurobiology, Department of Biotechnology, University of Rijeka, Rijeka (Croatia)

- working on Master thesis within HRZZ Research Project "Exploring the borderland between neurodegeneration and neuroregeneration: identification of key molecules with proteomics and functional assays in the mammalian spinal cord"

Principal Investigator: Miranda Mladinić Pejatović (2017.)

- cell cultures

-immunofluorescence

(immunocytochemistry and immunohistochemistry)

01/10/2018–10/11/2019

Student demonstrator

Department of biotechnology, University of Rijeka, Rijeka (Croatia)

- helped before and during laboratory exercises

- course *Immunology,* undergraduate programme "*Biotechnology and drug research*"

01/10/2017–08/11/2018

Student demonstrator

Department for biotechnology, University of Rijeka (Croatia)

- helped before and during laboratory exercises

- course *Immunology,* undergraduate programme "*Biotechnology and drug research*"

10/07/2017–21/07/2017

Student Intern

Jadran Galenski Laboratorij, Rijeka (Croatia)

JGL mentor Zrinka Badurina Huljev

Acquired skills during my internship at the Department for Research and Development

- calibration of pH-meter and scale
- assesment of: osmolality, particle size, density, pH value, and viscosity

PERSONAL SKILLS

Mother tongue(s): Croatian

Foreign language(s)

	UNDERSTANDING		SPEAKING		WRITING
	Listening	Reading	Spoken interaction	Spoken production	
English	C1	C1	C1	C1	C1
Italian	B2	B2	A2	A2	B1

Levels: A1 and A2: Basic user - B1 and B2: Independent user - C1 and C2: Proficient user
Common European Framework of Reference for Languages

ADDITIONAL INFORMATION

Additional education

Summer school in Pathophysiology and Public Health; St. Cloud State University/University of Rijeka (Rijeka, Croatia), June 2019

Scholarships

- Erasmus+ grant for an internship, University in Rijeka/University of Trieste, 2019./2020.
- City of Rijeka scholarships for gifted secondary school pupils and students, academic year 2015./2016., 2016./2017., 2017./2018. and 2018./2019.

Honours and awards

- 3rd prize in the Science category at the STEM games competition, Lanterna, May 2019.
- 2nd prize in the Science category at the STEM games competition, Lanterna, May 2018.
- honor student, Rijeka 2010.

Conferences

- active participant at the Young Neuroscientists Meeting 2019, International School for Advanced Studies (SISSA), Trieste, Italy

oral presentation: Expression of the CNS markers in primary neuronal cultures of neonatal opossums

- active participant at the Student Congress of Neuroscience NeuRi 2018., Rijeka - Rab, Croatia

oral presentation: Molecular basis of schizophrenia

Projects

- participation in the HRZZ Research Project "Exploring the borderland between neurodegeneration and neuroregeneration: identification of key molecules with proteomics and functional assays in the mammalian spinal cord", Laboratory for Molecular Neurobiology, principal Investigator Miranda Mladinić Pejatović, 2018./2019. godina
- participation in The Student mentor Project, Department of Biotechnology, academic year 2015./2016., 2016./2017. and 2018./2019.
- participation in the project "DVD pokusi za državnu maturu" in collaboration with publishing house Školska knjiga, academic year 2017./2018. and 2018./2019.

Memberships

- member of Biotechnology Students Association Rijeka

Volunteering

- volunteer at various humanitarian events (i.e. RijekaRun - humanitarian marathon)
- lecturer at the Rijeka Science Festival, Rijeka 2017.- 2019. godine
- scientific presenter at Student Day Festival, Rijeka 2017. - 2019.
- lecturer on the project "Summer School of Chemistry SobRi", Rijeka 2017. & 2018.
- volunteer at WUC Handball, Rijeka 2018.
- volunteer at European Researchers' Night, Rijeka September 2018.