IDENTIFICATION OF MITOTICALLY COMPETENT SOX2+ CELLS IN WHITE MATTER OF NORMAL HUMAN ADULT BRAIN

Oliver-De La Cruz J[1], Carrión-Navarro J[1], Gutiérrez-Martín A[2], Escobedo-Lucea C[3], García-Verdugo JM[1,3] and Ayuso-Sacido A[1]

[1] Regenerative Medicine Area. Centro de Investigación Príncipe Felipe and RETICS-CIBERNED. 46012 Valencia, Spain.

[2.] Department of Neurosurgery. Hospital Universitario la Fe, Valencia, Spain.

[3] Cell Morphology Department, Instituto Cavanilles. Universidad de Valencia, Valencia, Spain.

Corresponding authors

Angel Ayuso-Sacido, PhD

Regenerative Medicine Area

Centro de Investigación Príncipe Felipe

AVDA Autopista del Saler 16

46012, Valencia (Spain)

Tf: +34 963289680

e-mail: aayuso@cipf.es

Jose Manuel García-Verdugo, PhD

Department of Cell Morphology

Centro de Investigación Príncipe Felipe

AVDA Autopista del Saler, 16

46012, Valencia (Spain)

Tf: +34 963289680

e-mail: aayuso@cipf.es

***SOX2* expression is linked to the undifferentiated state of stem cells in mammalian neurogenic niches. While its expression has been reported in the adult human subventricular zone (SVZ), to date it has not been detected in adult human white matter. Here we describe a population of SOX2$^+$ cells from the white matter of the adult human temporal lobe, which proliferate and express glial markers *in vitro*.**

The transcription factor SRY (sex determining region Y)-box 2, also known as *SOX2*, is a member of the HMG-box superfamily of DNA-binding proteins. These genes play critical roles in self-renewal and cell fate decisions during early embryogenesis[1], fetal development, and neuroectoderm formation[2]. SOX2 function is also necessary for maintenance of the undifferentiated state of stem cells in adult mammalian neurogenic niches[3, 4]. Although SOX2 is reportedly expressed in the human SVZ[5], it has not been detected in normal adult white matter, a potential source of oligodendrocyte progenitor cells[6, 7]. White matter samples can be obtained from patients undergoing temporal lobe resection for drug-resistant partial epilepsy; when they are acquired at a suitable distance from the epileptic focus and without deterioration due to illness, they are considered the best source of normal samples obtained from living patients[6]. In these experiments, we focused on the isolation and identification of glial progenitor cells from adult human brain parenchyma, explored their potential *in vitro*, and identified their location in the original tissue.

Human temporal lobe samples were obtained from patients with intractable epilepsy (age, 17-58 years) who consented to tissue use under protocols approved by Neurosurgery Department, Hospital La Fe (Valencia, Spain).

In our initial experiments, cell isolation was accomplished using a protocol described previously[6, 8]. We established 4 adherent primary cultures (Fig. 1a) from 14 samples (success rate ~30%), which could be propagated as a monolayer culture for a minimum of 7 passages. We observed a diminishing proliferation rate and changes in initial bipolar cell morphology through subsequent passages.

To improve the efficiency of the isolation protocol, a final washing step was included as described in Johansonn *et al* (1999)[9]. Briefly, dissociated cells were resuspended in 0.9 M sucrose in 0.5× Hank's balanced salt solution (HBSS) without Ca^{2+} and Mg^{2+}, and centrifuged at 750 *g* for 10 min to remove myelin and cell debris. Using this method, we derived primary cultures from all samples processed (6 of 6). These cultures grew as floating or slightly adherent neurospheres instead of adherent cultures (Fig. 1b). We obtained tertiary neurospheres (at least) from all cultures, confirming self-renewing capacity, although both their number and size decreased with increasing passage number. In addition, these neurospheres generated an adherent culture in each passage that could be propagated separately through at least 5 passages (figure 1c-d).

We selected 3 cell lines from each type of culture for molecular characterization by PCR with a battery of primers designed for the detection of neural stem cells and differentiation markers (see Supplementary Table 1). The analysis revealed distinct expression patterns of stem cell markers (Fig. 1e) among the 3 cell types, whereas expression was equivalent within each set of lines (Supplementary Fig. 1). While both types of adherent culture showed a very similar profile, neurospheres differed from adherent lines in the absence of *ABCG2*, *MSI1*, and *PAX6*, the presence of *CD133* and, notably, higher expression of *SOX2*.

This *SOX2* enrichment in neurospheres was confirmed by qRT-PCR analysis. *SOX2* expression levels were more than 1000-fold higher in neurospheres than in the original adherent cultures, and about 100-fold higher than in the adherent lines derived from the neurosphere cultures (Fig. 1f).

SOX2 protein was detected by immunocytochemistry only in neurospheres (Fig. 1h–k, Supplementary Fig. 2), in which $SOX2^+$ cells comprised 80.57% ± 17.20% of the total cell number. Interestingly, $SOX2^+$ cells are already present in initial passages, although in lower numbers (43.69 ± 8.92%). Co-immunostaining with Ki67 revealed that all $Ki67^+$ cells in neurospheres were $SOX2^+$.

The molecular analysis also demonstrated that the enrichment of $SOX2^+$ cells was coincident with changes in glial gene expression (Fig. 1f). In addition to *NG2* and *PDGFRA*, which were also detected in the original adherent cultures, neurospheres expressed a higher number of both earlier and later oligodendrocyte differentiation markers. These included the transcription factors *OLIG1* and *OLIG2*, and *MAG*, *PLP*, and *MBP*, all encoding myelin proteins. The expression of A2B5 and O4, earlier oligodendrocyte markers, was also confirmed by immunocytochemistry (Supplementary Fig. 3) in neurospheres, but was undetectable in the adherent lines.

The discovery of this $SOX2^+$ population of white-matter derived cells led us to examine its source in 4 samples of adult human temporal lobe white matter. Tissues were fixed for 48 h in 4% paraformaldehyde, and 12-µm sections were cut on a cryostat. Samples showed no evidence of pathology (Supplementary Fig. 4). Sections of human glioblastoma expressing high levels of SOX2 served as positive controls (Supplementary Fig. 5)

Immunohistochemical studies detected $SOX2^+$ cells in all samples analyzed at a very similar number, constituting less than 2% of the total cells. Such cells were scattered throughout the white matter, sometimes concentrated in small areas. No proliferating $SOX2^+$ cells were detected using Ki67 co-immunostaining (data not shown).

We performed co-immunostaining with neuronal and glial markers to determine the $SOX2^+$ cell phenotype. We observed no co-staining with Iba-1 (Fig. 2 a–d),

CNPase (Fig. 2e–h), or NeuN, (Fig. 2i–l), suggesting that the SOX2$^+$ cell subpopulation does not correspond to a differentiated microglial, oligodendroglial, or neuronal population. However, GFAP immunohistochemistry revealed co-immunolocalization with SOX2$^+$ in a small population of cells. Although it is difficult to estimate SOX2$^+$/GFAP$^+$ cell frequency due to intense GFAP immunoreactivity in the white matter, both GFAP$^+$ (Fig. 2m–p) and GFAP$^-$ (Fig. 2q–t) cells were identified in all samples. The SOX2 staining pattern clearly differs from that seen in animal models, in which all astrocytes throughout the parenchyma express Sox2[4]. This contrast, along with other differences such as unique SVZ functional organization, could explain lower glial tissue regeneration observed in the adult human brain[7, 10].

We have obtained an enriched SOX2$^+$ cell population *in vitro* after adding a washing step with sucrose to the isolation protocol. Similar results using a Percoll density gradient purification step were obtained using adult rat SVZ-derived neural progenitor cells[11]. Alternatively, recently described approaches for SOX2$^+$ cell enrichment from fetal tissue using adenoviral transduction[12] could be used for direct isolation of SOX2$^+$ cells from adult human white matter.

Moreover, our findings provide the first evidence that SOX2$^+$ cells exist not only in neurogenic niches or neoplastic tissues[13], but also in normal adult white matter. We saw no co-immunolocalization with neuronal or oligodendroglial markers, and only a small fraction of SOX2$^+$/GFAP$^+$ cells was detected. Thus, as it occurs in the SVZ where SOX2 is known to be expressed in glial progenitors[5], we hypothesize that these SOX2$^+$ cells are also a quiescent or "sleeping" glial progenitor cell cohort that could be activated after brain injury in an attempt to repair damage. This idea is also supported by *in vitro* results, where the detection of SOX2, the absence of neuronal compromised cell markers like *PAX6*[5], and the enhanced expression of oligodendrocyte and astrocyte markers, reveal an enrichment of glial progenitor cells in our cultures. These white-matter derived cultures provide an *in vitro* model for further characterization of their proliferative signals and differentiation potential, and can facilitate attempts to modulate their activity *in vivo*.

BIBLIOGRAPHY

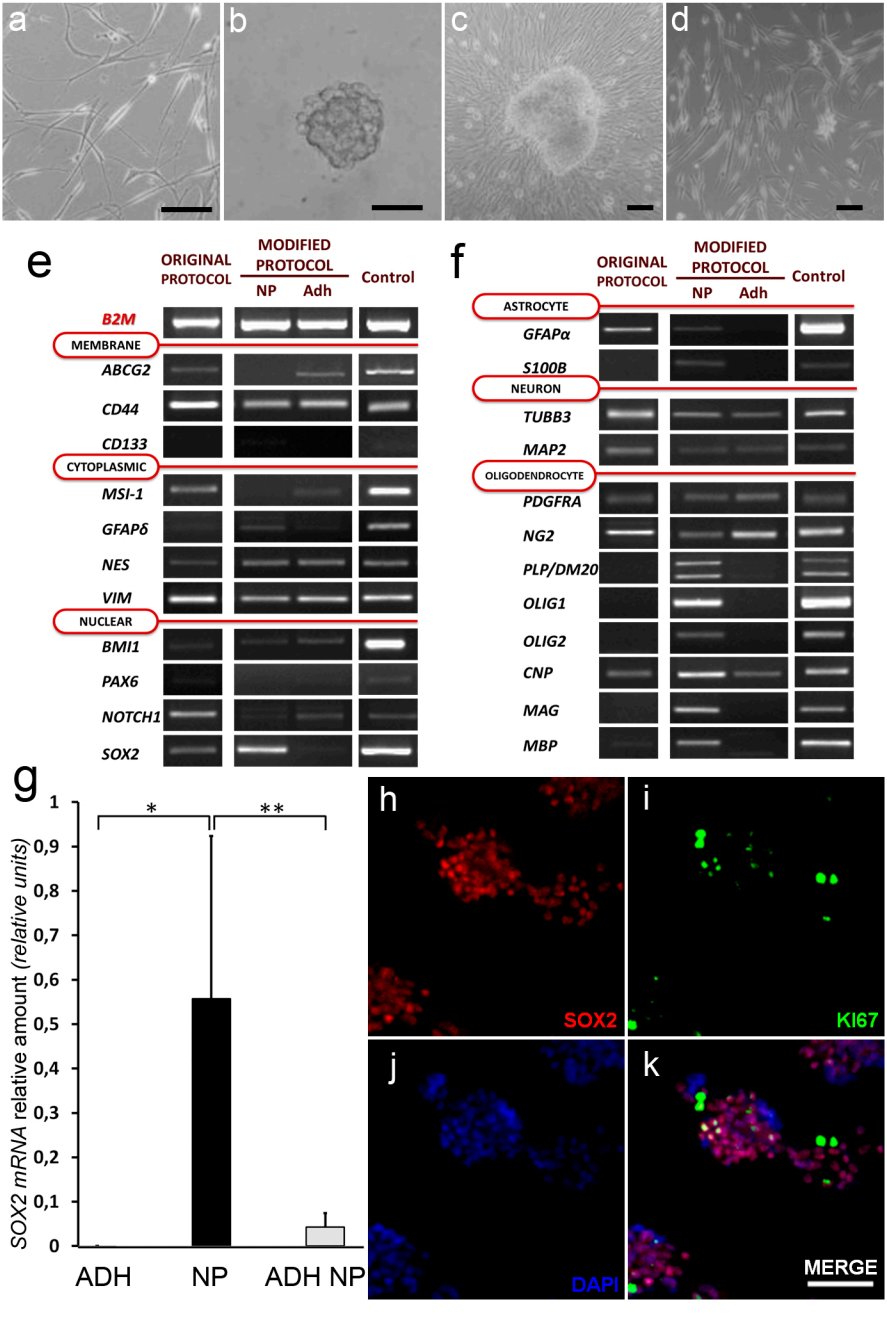
1. Avilion, A.A. *et al. Genes Dev* **17**, 126-140 (2003).
2. Papanayotou, C. *et al. PLoS Biol* **6**, e2 (2008).
3. Ellis, P. *et al. Dev Neurosci* **26**, 148-165 (2004).
4. Komitova, M. & Eriksson, P.S. *Neurosci Lett* **369**, 24-27 (2004).
5. Baer, K., Eriksson, P.S., Faull, R.L., Rees, M.I. & Curtis, M.A. *Exp Neurol* **204**, 828-831 (2007).
6. Scolding, N.J., Rayner, P.J., Sussman, J., Shaw, C. & Compston, D.A. *Neuroreport* **6**, 441-445 (1995).
7. Scolding, N. *et al. Brain* **121 (Pt 12)**, 2221-2228 (1998).
8. Ayuso-Sacido, A., Roy, N.S., Schwartz, T.H., Greenfield, J.P. & Boockvar, J.A. *Neurosurgery* **62**, 223-229; discussion 229-231 (2008).
9. Johansson, C.B. *et al. Cell* **96**, 25-34 (1999).

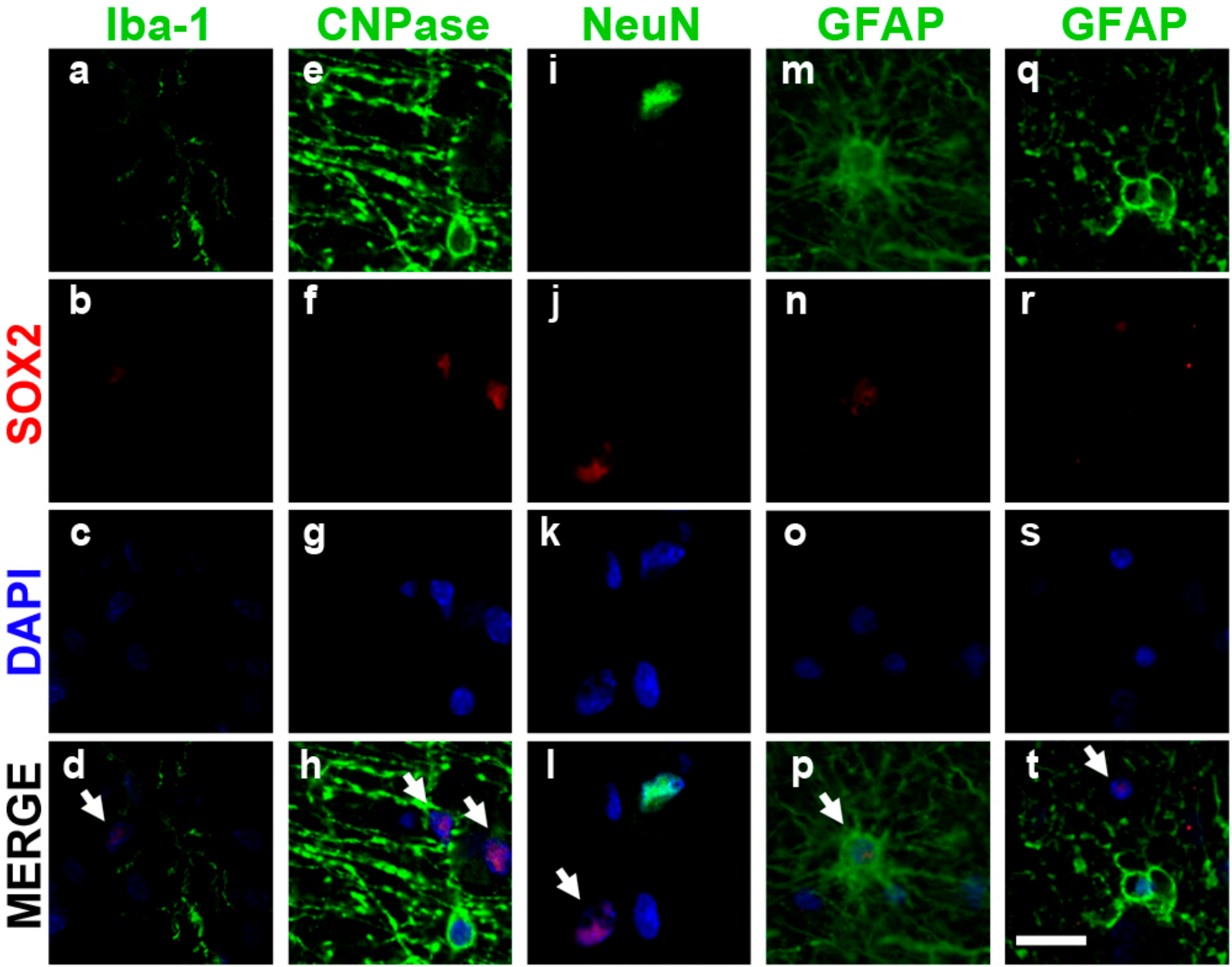
10. Patrikios, P. *et al. Brain* **129**, 3165-3172 (2006).
11. Chen, K., Hughes, S.M. & Connor, B. *Cell Transplant* **16**, 799-810 (2007).
12. Wang, S. *et al. J Neurosci* **30**, 14635-14648 (2010).
13. Phi, J.H. *et al. Am J Surg Pathol* **32**, 103-112 (2008).

FIGURE LEGENDS

Figure 1: Progenitor cells can be isolated from adult human white matter. Original protocol gives rise to monolayer culture (**A**), whereas the modified protocol produced neurospheres (**B**) which generate an adherent culture (**C**) that could be passaged separately (**D**). Scale bars represent 10 um. **E-F:** molecular characterization by PCR of stem cell and neural lineage markers was performed for the three types of cell lines. Primers, band size and positive controls can be consulted at supplementary figure 1. PCR from all samples considered can be consulted at supplementary figure 2. **G**. qPCR revealed that neurospheres (NP) express *SOX2* more than 1000-fold than cells from original protocol (ADH) (*P-value=0,0068< α=0,05) and about 100-fold more than adherent culture derived from them (ADH NP) (**P-value:0,0091<α=0,05). Statistical analysis was performed with Kruskal-Wallis test followed by Mann-Whitney comparisons with post hoc bonferroni correction. Data represents mean ± s.e. **H-K** Immunohistochemistry for SOX2 and KI67. SOX2 protein could only be detected in neurospheres. All $Ki67^+$ cells co-stain with SOX2. Scale bar 20um. Immunostaining for the rest of cell types can be consulted at supplementary figure 3.

Figure 2: Immunohistochemical characterization of $SOX2^+$ cells from adult temporal lobe white matter. No co-immunolocalization was detected for Iba-1(a-d), CNPase (e-h) or NeuN (i-l). Some $SOX2^+$ cells were GFAP + cells (m-p) but not all of them (q-t). Arrows indicate $SOX2^+$ cells. Scale bar represents 20 um. Immunostaining for glioblastoma sections used as positive controls are collected in Supplementary figure 5.


a
b
c
d
e
ORIGINAL PROTOCOL
MODIFIED PROTOCOL
NP
Adh
Control
B2M
MEMBRANE
ABCG2
CD44
CD133
CYTOPLASMIC
MSI-1
GFAPδ
NES
VIM
NUCLEAR
BMI1
PAX6
NOTCH1
SOX2
f
ORIGINAL PROTOCOL
MODIFIED PROTOCOL
NP
Adh
Control
ASTROCYTE
GFAPα
S100B
NEURON
TUBB3
MAP2
OLIGODENDROCYTE
PDGFRA
NG2
PLP/DM20
OLIG1
OLIG2
CNP
MAG
MBP
g
SOX2 mRNA relative amount (relative units)
1
0,9
0,8
0,7
0,6
0,5
0,4
0,3
0,2
0,1
0
*
**
ADH
NP
ADH NP
h
SOX2
i
KI67
j
DAPI
k
MERGE


Iba-1
CNPase
NeuN
GFAP
GFAP
SOX2
DAPI
MERGE
a
b
c
d
e
f
g
h
i
j
k
l
m
n
o
p
q
r
s
t