

Title:

Dynamic distribution and stem cell characteristics of Sox1-expressing cells in the cerebellar cortex.

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Running Title: Characteristics of Sox1⁺ cells in the maturing cerebellar cortex.

Keywords: Cerebellum, Sox genes, Stem cell.

ABSTRACT:

Bergmann glia cells are a discrete radial glia population surrounding Purkinje cells in the cerebellar cortex. Although Bergmann glia are essential for the development and correct arborisation of Purkinje cells, little is known about the regulation of this cell population after the developmental phase. In an effort to characterize this population at the molecular level, we have analysed marker expression and established that adult Bergmann glia express Sox1, Sox2 and Sox9, a feature otherwise associated with neural stem cells (NSCs). In the present study, we have further analysed the developmental pattern of Sox1-expressing cells in the developing cerebellum. We report that before becoming restricted to the Purkinje cell layer, Sox1-positive cells are present throughout the immature tissue, and that these cells show characteristics of Bergmann glia progenitors. Our study shows that these progenitors express Sox1, Sox2 and Sox9, a signature maintained throughout cerebellar maturation into adulthood. When isolated in culture, the Sox1-expressing cerebellar population exhibited neurosphere-forming ability, NSC-marker characteristics, and demonstrated multipotency at the clonal level. Our results show that the Bergmann glia population expresses Sox1 during cerebellar development, and that these cells can be isolated and show stem cell characteristics in vitro, suggesting they could hold a broader potential than previously thought.

INTRODUCTION:

Neural stem cells (NSCs) have been identified in 2 established areas of the vertebrate brain, the lining of the lateral ventricles and the dentate gyrus area of the hippocampus. Among the set of markers used to identify NSCs, expression of Sox1 and Sox2, two transcription factors from the SoxB1 group, is a molecular characteristic widely used to identify NSCs both in situ and in vitro. Members of the SoxB1 subgroup such as Sox1 and Sox2 are known to be involved in neural induction during embryogenesis. Sox1 plays a critical role in the development of neural precursors from undifferentiated embryonic stem cells [1, 2] and embryonal carcinoma cultures in vitro [3]. Sox1 and Sox2 remain expressed in the two recognised stem cell populations of the adult CNS, the subventricular zone (SVZ) and dentate gyrus (DG) of the hippocampus, and Sox1- and Sox2-expressing neural stem cells retain the ability to proliferate and differentiate into glial and neuronal derivatives in vitro and in vivo [4-6]. Because of this specific expression pattern, an increasing number of studies rely on the use of these markers to identify and isolate progenitors from mouse brain tissue [4] and from embryonic stem cell cultures [1, 7].

The SVZ and DG stem cell populations have been intensely studied since their discovery, but only recently have other areas of the adult brain been investigated for the presence of NSCs. Although the cerebellum was not traditionally thought to contain neuroprogenitors, several reports have suggested that it may harbour cells with stem cell characteristics [8, 9]. We have recently established that in the adult, expression of Sox1 and Sox2 is also present in Bergmann glia, which is located in the Purkinje cell layer, but absent from the white matter [10]. This discrete radial glia population of the adult cerebellum cortex retains expression of Sox1, Sox2 and Sox9, a property shared with established NSCs from the SVZ and the DG [10]. However, an independent study carried out in immature mouse cerebellum reported the presence of putative stem cells identified in the white matter [9]. In order to establish the precise location and nature of neuroprogenitors in the cerebellum and further analyse their potential, we have analysed the characteristics of Sox1-expressing cells in the immature cerebellum and after isolation in vitro. In this paper, we report the dynamic distribution of Sox1-expressing cells during cerebellar development until maturation, and provide in situ and in vitro data suggesting that this Sox1-positive cerebellar cell population exhibits stem cell-like properties so far unreported.

RESULTS:

Developmental distribution of Sox1-expressing cells in the developing cerebellum:

Following our initial observation that the mature cerebellum harbours a discrete population of Sox1-expressing cells, we have analysed different stages of cerebellum development to determine the distribution of Sox1-positive cells during tissue maturation. To this end we studied animals from the Sox1-GFP mouse line, known to provide a faithful GFP reporter signal indicative of Sox1 expression [10, 11]. Cerebella from Sox1-GFP^{+/-} mice were collected at stages ranging from birth (P0) to postnatal day 25 (P25), and the expression of the GFP reporter was analysed in tissue sections (Fig. 1). At P0 and P4 stages, GFP-positive cells were detected throughout the cerebellum except in the external granular layer (EGL). Sections of P7 cerebellum showed some evidence of differential distribution of Sox1-positive cells (Fig.1C,G), which appeared predominantly located in the Purkinje cell layer (PCL). This trend was confirmed at P15 and P18, when GFP cells became restricted to the PCL. By P25, no GFP expression could be found outside of the PCL. This dynamic distribution pattern was confirmed at the RNA level using RNA in situ hybridisation, which showed that by P25, expression of the GFP transgene is restricted to the Bergmann glia in the Purkinje cell layer (Fig. 1I) as previously observed in the adult. To further determine the nature of these Sox1-positive cells detected in the immature cerebellum, we analysed expression of BLBP and S100, known to mark Bergmann glia cells [12, 13]. Results obtained in sections of P7 cerebellum showed significant overlap for the expression of the GFP reporter with S100 (Fig. 2A-C) and with BLBP (Fig. 2D-F) particularly visible in the PCL, thus indicating that the Sox1-expressing population found in the immature cerebellum are predominantly found to express S100 and BLBP, in line with a Bergmann glia lineage identity described in the mature tissue.

Having previously shown that Sox2 and Sox9 are expressed in the Sox1-positive cell population of the adult cerebellum, we proceeded to determine whether co-expression of these 3 genes was also seen before cerebellar maturation by analysing early postnatal stages. In situ hybridisation with antisense riboprobes for Sox2 (Fig. 3A-D) and Sox9 (Fig. 3E-H) indicated a dynamic expression pattern similar to that observed for Sox1, with widespread expression getting progressively restricted to the Purkinje cell layer as the cerebellum matures. Although some differences were perceptible between the detection of mRNA (Sox2 & 9) and protein (GFP) expression (possibly due to differences in target stability in the cell), the pattern of

GFP signal analysed on the same tissue sections suggested overlapping expression of Sox2 and Sox9 with Sox1 throughout the postnatal developmental phase. Parallel analysis of Sox3 expression by in situ hybridisation produced a weak signal at P4, which could not be detected above background at later stages (data not shown).

To determine whether the dynamic distribution of Sox-positive cells in the cerebellum is a conserved feature, we analysed tissue from chick brains collected at late stages of development (HH stages 40-44), which correspond to early postnatal stages of mouse CNS maturation [14]. Expression of Sox1, Sox2 and Sox9 was detected in the cerebellar cortex (Suppl. Fig. 1), in a similar pattern to that seen in maturing mouse cerebellum, with strong Bergmann glia staining and residual signal in the white matter and IGL, suggesting a similar dynamics of expression to that observed in mouse tissue.

In vitro characterisation of Sox1+ cells isolated from the cerebellum:

In order to compare the Sox1-expressing fraction observed in P7 cerebellum to a progenitor population previously reported in the immature tissue [9], we analysed the expression of cd133 in a P7 cerebellum cell suspension. FACS analysis revealed that 90.1% of cd133-positive cells in P7 cerebellum were GFP-positive in the Sox1-GFP model (Suppl. Fig2). In order to further analyse the properties of the Sox1-positive population in mature cerebellum, cerebella were collected from Sox1-GFP^{+/+} mice and the tissue was disaggregated. Due to the highly organised and integrated structure of mature cerebellar tissue, a combination of enzymatic and mechanical methods was required to obtain a cell suspension. Although significant cell death was observed, a cell suspension could be analysed by flow cytometry, revealing a significant GFP-positive fraction (Fig. 4B). Tissue from the lateral ventricle was used to isolate GFP-positive NSCs used as positive control, whereas GFP signal was seen to be duly absent from samples isolated from wild type littermates used as negative controls.

The numbers of viable cells surviving the isolation and flow cytometry protocol from fresh tissue were consistently and critically low at the end of the procedure, and cells collected after this process consistently failed to grow when subsequently placed in culture. It seemed probable that the specific structure of the mature cerebellum, and the elaborate morphology of the BG layer observed in the adult with its long radial processes intertwined with Purkinje arborisations [13, 15], make it particularly sensitive to tissue disruption protocols. In order to allow the isolation of GFP-labelled cells and the establishment of a viable culture, an

alternative procedure was developed exploiting the expression of the GFP-ires-Pac cassette present in Sox1-GFP^{+/-} animals. Since the expression of this construct downstream of the endogenous Sox1 promoter [11] confers puromycin resistance to Sox1-expressing cells, the application of puromycin selection to cerebellar samples collected after enzymatic treatment provided an alternative to mechanical disruption in order to gradually break down the tissue, allow the progressive release of individual cells, and promote the selective culture of Sox1-expressing cells. Cell suspensions from Sox1-GFP^{+/-} cerebella were maintained for 6 days in medium containing puromycin to selectively eliminate non-Sox1 expressing cells from the culture. The selection was subsequently removed from the culture medium, the cell suspension was washed and replated in fresh medium, and refractile aggregates were observed floating in the culture after 7 days. These aggregates grew in size over time, forming structures reminiscent of neurospheres (Fig. 4E,F). These cerebellar-derived neural stem-like cells ('CB-NLCs') were subsequently passaged for further characterisation. No aggregates were detected after similar treatment of samples isolated from wild type littermates, confirming the efficiency of the puromycin-induced selection in the absence of the transgene. Such aggregates were observed in preparations of wild type cerebellum in the absence of puromycin (data not shown), however the lack of selection severely limited the efficiency of tissue disaggregation in culture, making the process very inefficient.

Characterisation of cerebellum-derived NSC-like cells:

CB-NLC cultures were very similar to control NSCs, although they displayed a greater affinity for tissue culture-treated plastic when reaching high cell density. The population doubling time of CB-NLCs was calculated and shown to be slightly higher (2.8 days) than that of NSC cells (2.1 days). CB-NLCs maintained in culture were routinely passaged twice a week, and showed clear morphological resemblance to control NSC cultures isolated from lateral ventricle tissue. Flow cytometry analysis of Nestin and Sox2 expression established strong positive staining of the CB-NLC population, as observed in control NSC culture, as well as cd133 expression (Fig. 5A). Further phenotypical analysis showed positive immunostaining for A2B5, Islet1, Vimentin, cd133, Blbp, Nestin, Sox2 and Sox9, all markers associated with NSCs (Fig. 5B).

The gene expression pattern of CB-NLCs was analysed by RT-PCR and compared to the NSC population (Fig. 5C). Analysis of the RT-treated samples and corresponding '-RT' controls was used to detect

transcripts from intronless genes such as Sox1, 2 and 3, and confirmed the expression of Sox1 and Sox2, and the absence of signal for Sox3 in both populations (Fig. 5C). Sox2 signal appeared stronger than Sox1, and expression of Sox1 was further confirmed by the detection of the GFP reporter transcript. In addition, PCR analysis with primers designed to amplify across introns allowed the detection of cDNA for NSC markers including ABCG2, Rest, Pax6, Glast1, Olig2, and Msi1&2. RT-PCR also confirmed the expression of Sox9, Nestin, Blbp and cd133, and showed the absence of detectable Oct4 expression in the samples analysed.

In order to further examine the stem cell potential of cells from the neurosphere-like structures at the cellular level, aggregates isolated from adult cerebellum were treated with accutase and single cells were seeded into wells of 96-well plates using a FACS sorter (Fig. 6). After 10 days in culture, clonal cultures were visible (calculated frequency of 5.2%), and were picked for expansion and further characterisation. In vitro analysis by immunostaining showed expression of nestin and Sox2 in resulting clonal cultures (Fig. 6B), a result confirmed by flow cytometry analysis, which also established expression of cd133 and ABCG2 (Fig. 6C). The potential of clonal cultures was analysed in response to differentiation culture conditions, which triggered a change in cell morphology and appearance of GFAP-, CNPase- and β 3Tubulin-positive derivatives (Fig 6D), thus establishing the self-renewal and multipotent properties of CB-NLCs at the clonal level.

DISCUSSION:

The Bergmann glia is a radial glial population residing in the Purkinje cell layer, with cell bodies located adjacent to Purkinje neurons and radial processes extending to the pial surface. Little is known about the developmental origin and biological functions of Bergmann glia beyond their documented scaffolding role for both the guidance of Purkinje cell dendrites and the migration of granule cell precursors [13, 15, 16]. We had previously shown that the two neural stem cell markers Sox1 and Sox2 are co-expressed together with Sox9 in the Bergmann glia population located in the Purkinje Cell layer (PCL) of the adult mouse cerebellum [10, 17], and more recently in the human cerebellum [18]. The present study demonstrates that Sox1-expressing cells are present outside of the PCL at early postnatal stages, a result in line with a previous report that Sox1-expressing progenitors are marked by labelling in the immature white matter [19]. Sox1 expression was detected in a pattern similar to that of Sox2 and Sox9 and observed throughout the cerebellum (except the EGL) in early postnatal stages, before being progressively restricted to the Purkinje cell layer as the tissue matured. This cerebellar pattern was also observed in immature chick tissue. As the cerebellum completed its postnatal maturation, Sox1 expression was only seen in the Bergmann glia population.

Our study shows that, in the immature cerebellum, Sox1 expression is widespread across all tissue layers from the white matter to the molecular layer. This suggests a migration path of Sox1-expressing cells, from the ventricular zone towards the cortical layers, which is consistent with that of Bergmann glia precursors during cerebellar formation [13, 20], although the possibility of Sox1 expression being sequentially turned off and on in different populations during development cannot be formally excluded at this stage. Although an earlier report proposed that Sox1 expression marked a neuronal population [19], the results presented here show that the Sox1-positive cells express Sox9, BLBP and S100, a signature recently confirmed to be associated with Bergmann glia progenitors [12, 21]. These observations suggest that Sox1 / Sox2 / Sox9 expression, typically associated with neural stem cells in the CNS, is also a characteristic of Bergmann glia during and after cerebellar maturation.

The pattern of Sox1-expressing cells observed at P7 would explain the apparent contradiction between the presence of a stem cell-like population located in the white matter of the immature cerebellum, as reported

by Lee et al. [9], and recent evidence that expression of the stem cell markers Sox1/Sox2/Sox9 are specifically found in the Purkinje cell layer in the adult [10]. Cells identified by Lee et al. in P7 white matter were shown to be positive for Sox2, Musashi-1, Nestin and cd133, however no data was available on the Sox1 status of this cell population. Interestingly, we have found that cd133-positive cells present in P7 cerebellum are positive for the Sox1 reporter, thus supporting a significant overlap between the cells observed by Lee et al. in the P7 white matter and the Sox1-expressing population described here.

Since expression of Sox1/Sox2/Sox9 is associated with neural stem cells in other areas of the brain [10], their expression in the cerebellum suggested stem cell potential in the Sox1/Sox2/Sox9-positive Bergmann glia. The radial glia phenotype of Bergmann glia would support this hypothesis, since robust evidence links radial glia and stem cell potential in the CNS and radial glia cells can generate neuronal and glial derivatives [22-24]. When isolated in culture, the Sox1-expressing cell population showed phenotypic characteristics, clonogenicity and a differentiation potential consistent with a neural stem cell potential. Another recent study also reported that cells with neurosphere-forming ability could be found in tissue disaggregated from adult cerebellum [8], in line with our observation; however the isolation procedure used in this early report did not provide any indication on the origin, localisation or nature of the isolated cell population. The isolation method described here, with the in vitro selection step, differs from that used in the report by Klein et al, so it remains to be established whether the resulting cell populations described in both studies are identical or not. Results produced in the present study support the Bergmann glia as the source of this stem cell activity present in the cerebellum, and confirm that Sox1 expression may represent a unifying criterion to identify NSC-like cells present throughout the CNS.

More experiments are now needed to evaluate the stem cell characteristics of Bergmann glia in an in vivo setting. Bergmann glia have not so far been reported to have any stem cell ability, and it will therefore be critical to analyse in more details whether self-renewal and differentiation are observed in the tissue. There is evidence showing that the quiescent Bergmann glia population can be activated in vivo in response to local damage. There are many reports in the literature describing the occurrence of Bergmann glia proliferation in response to damage in a range of models [25-28]. It is also established that although expression Nestin, another NSCs marker, is not detected in adult cerebellum ([29] and data not shown), it

can be reversibly activated in Bergmann glia following disruption of the Purkinje Cell Layer [25, 30, 31]. Similarly, induced expression of PSA-NCAM and cd133 has recently been observed in a model of cerebellar damage [25]. These observations suggest that the Bergmann glia is more plastic than initially thought. It is also possible that the culture protocol used to isolate and grow the cells in culture may be critical to revealing a stem cell phenotype, despite a more limited potential attributed to the cells in situ [25]. Our data show that the Sox1-positive cerebellar population is able to exhibit stem cell characteristics when subjected to standard NSC culture conditions. It is possible that the multipotency and self-renewal ability of these cerebellar cells *ex vivo* may be dependent on exposure to one (or more) stimulus provided during the *in vitro* process, whether through the effects of tissue disruption or the presence of mitogens in culture. These observations may thus reflect the ability of Sox1-positive cells to respond to a change in the properties of their environment or niche, which is known to play a central role in controlling the fate of quiescent stem cells in the brain [32, 33].

These findings on Bergmann glia are notable in the light of the recent report describing the multipotency of quiescent cortical astrocytes. These cortical glial cells, which can also undergo reactive gliosis *in vivo*, have been shown to form multipotent neurospheres *in vitro* even though their *in vivo* potential appears more limited [34]. The fact that mature Bergmann glia can also change from quiescent state to reactive gliosis in response to injury, together with our observation that these cells can display NSC characteristics *in vitro* thus appears to support the hypothesis that reactive glial cells represent a broad source of multipotent progenitors [34]. These observations highlight the importance of carrying out a thorough analysis of Sox1 & Sox2 expression in the developing and mature CNS to both identify all cell populations sharing these molecular characteristics, and conclusively disentangle the link between Sox1 and Sox2 expression and stemness *in vivo*. More experimental evidence is also needed to evaluate whether Sox1-positive Bergmann glia can display NSC characteristics *in vivo*, and whether their response to cerebellar injury involves the activation of a stem cell component which could be of therapeutic value for endogenous tissue repair.

MATERIAL AND METHODS:

Materials were purchased from Invitrogen (Paisley, UK) unless otherwise stated.

Cell culture preparation:

Heterozygous Sox1-GFP^{+/-} mice, which carry a knocked in GFP reporter [11], were sacrificed by cervical dislocation (in accordance with the UK Animals (Scientific Procedures) Act 1986). The cerebellum was isolated, washed in PBS, and the tissue was minced with a razor blade. For control NSC cultures, tissue from lateral ventricles was collected and processed in parallel. Initially, the tissue was transferred to a conical tube, pipetted up and down through a glass Pasteur, washed in PBS and incubated in Accumax (Patricell Ltd, Nottingham, UK) for 45 minutes at 37°C. To improve viability, the mechanical disruption step was subsequently omitted and the Accumax incubation reduced to 30 minutes [35, 36]. In both cases, the resulting suspension was briefly centrifuged and washed in PBS before transferring to a 6-well plate containing NSC medium (see below). The plate was placed in an incubator (37°C, 5% CO₂) and fresh medium added after 3 days. The cultures were washed in PBS after 6 days to remove cell debris, and transferred to a fresh plate. A fraction of this initial culture was used for the single cell seeding experiment, while the remaining cells were replated to establish a stock (non-clonal) CB-NLC culture.

NSC medium was prepared with DMEM/F12 and Neurobasal medium (1:1), N2, B27, Pen/Strep, bFGF (20 ng/ml) and EGF (20 ng/ml, Sigma, Poole, UK). 0.5 µg/ml Puromycin (Sigma) was used during the selection phase as described in the text. Population doubling times were determined by serial plating of the cells at a constant density (5x10⁵ cells/well) and cell counting every 48h to generate cumulative population doubling curves used as described elsewhere [37].

NSC Culture:

To passage the cultures, the neurosphere suspension was transferred to a conical tube containing an equal volume of PBS, and pelleted by brief centrifugation (3 minutes, 200 g). The supernatant was replaced with 1 ml of Accutase (Patricell) and the sample was incubated at 37°C for 5 minutes. After a PBS wash, the pellet was resuspended in fresh medium and transferred to a new plate. Cultures were routinely split 1 in 3. For in vitro differentiation, NSC medium was replaced with medium containing DMEM/F12 and

Neurobasal (1:1), 1% FCS, Pen/Strep. Fresh medium was added every 2 days, taking care not to disturb the monolayer. Changes in cell morphology were visible within 2 days, and after 7 days cells were fixed with 4% ice-cold paraformaldehyde and stored at 4°C until analysis. For immunostaining, cells were seeded on glass coverslips coated with 0.1% gelatine (Sigma) solution to promote adhesion. After treatment, cells were fixed with 4% ice-cold paraformaldehyde and stored at 4°C until analysis.

Non-radioactive in situ hybridisation:

Brains isolated from Sox1-GFP^{+/+} mice were fixed in 4% paraformaldehyde overnight at 4°C and processed as previously described [10] to generate 10 µm tissue sections. Briefly, sections were treated with Proteinase K (Roche, Burgess Hill, UK), fixed again and incubated overnight at 70°C in hybridisation solution containing the DIG-labelled probe. Sections were then extensively washed, incubated 1 hour in blocking agent (Roche), and then overnight at 4°C with anti-Dig antibody (Roche). After extensive washing, the colour was developed using an NBT/BCIP reaction mix (Roche).

Immunohistochemistry and flow cytometry:

Samples were washed in PBS + 0.1% Tween-20 (Sigma), blocked for 1 hour in 1% blocking reagent (Roche), and incubated with a primary antibody diluted in 1% blocking reagent overnight at 4°C. After extensive washing in PBS + 0.1% Tween-20 for 40 minutes, samples were incubated in secondary antibody for 1 hour, washed for 1 hour in PBS + 0.1% Tween-20, and mounted with Vectashield medium containing dapi (Vector Labs, Peterborough, UK). Antibodies used were: anti-calbindin antibody (Sigma), anti-S100 and anti-GFAP antibodies (Dako, Ely, UK), anti-BLBP, anti-β3Tubulin, and anti-A2B5 antibodies (Millipore, Watford, UK), anti-nestin, anti-Vimentin and anti-Isl1 antibodies (Developmental Studies Hybridoma Bank, Iowa City, USA), anti-CNPase, anti-GFP and anti-cd133 antibodies (Abcam), anti-ABCG2 antibody (Santa Cruz Biotechnologies, Heidelberg, Germany), and appropriate conjugated secondary antibodies (VectorLabs). For Fast Red staining, a biotinylated secondary antibody (VectorLabs) was used, followed by a 1 hour incubation in alkaline phosphatase-conjugated streptavidin (VectorLabs) before using the Fast Red detection kit (Sigma) for the colour reaction.

For FACS analysis, cells harvested using Accutase were pelleted by centrifugation 5 minutes at 200 g, and fixed in 3 ml of 4% ice-cold paraformaldehyde for 20 minutes. The fixative was washed in PBS + 0.1%

Tween-20, and replaced with 0.5 ml of blocking solution for 30 minutes before incubation with the primary antibody (1/200) for 1 hour. Samples were washed 3 times in PBS + 0.1% Tween-20, and incubated in secondary antibody solution diluted in PBT (1/500) for 30 minutes. Samples were washed 3 times in PBS and stored on ice until FACS analysis on a Beckman-Coulter Altra flow cytometer.

RT-PCR:

Reverse transcription and PCR analysis were performed following protocols described elsewhere [38] (primers and PCR cycling parameters available upon request) using β actin and clathrin primers as loading controls.

ACKNOWLEDGEMENTS:

We are grateful to our colleagues N. Lane and Dr A. Robins for expert advice on flow cytometry, and we wish to thank Dr H. Priddle (U. of Nottingham) for critical reading of the manuscript, and Dr M. Li (Imperial College London) for kindly providing access to the original mouse line. JA was funded by a grant from the BRC (University of Nottingham). VS is indebted to the Anne McLaren fellowship scheme (University of Nottingham) and to the Alzheimer's Society for their support, past and present.

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FIGURE LEGENDS:

Figure 1: Dynamic distribution of Sox1-positive cells monitored at various stages of postnatal development in Sox1-GFP^{+/-} mouse cerebellum. GFP fluorescence signal (green) highlights the Sox1-positive population and the anti-calbindin immunostaining (red) marks Purkinje cells in sagittal sections of P0 (A), P4 (B), P7 (C), P15 (D), P18 (E) and P25 (F) cerebellum. Initially present throughout the immature cerebellum, GFP-positive cells progressively disappears from internal tissue layers ; by P25 GFP is only detected in Bergmann glia cells located in the Purkinje cell layer, where they are intercalated between Purkinje cells. Expression of the Sox1-GFP transgene in the white matter at P7 was confirmed by in situ hybridisation for Sox1 (G). Its expression is subsequently restricted to the Purkinje cell layer as shown by in situ hybridisation analysis of GFP expression at P15 (H) and P25 (I). ML: molecular layer, WM: white matter, IGL: internal granule layer, PCL: Purkinje cell layer, EGL: external granular layer. Bar=50 µm.

Figure 2: Double immunostaining on sagittal sections of P7 Sox1-GFP^{+/-} mouse cerebellum showing expression of Sox1 (A, green) and S100 (B, red), and Sox1 (D, green) and BLBP (E, red). (C,F) Composite overlay pictures presented to illustrate areas of overlapping expression (yellow, arrows). ML: molecular layer, WM: white matter, IGL: internal granule layer, PL: Purkinje cell layer. Bar=50 µm.

Figure 3: Changes in Sox2 (A-D) and Sox9 (E-H) expression analysed by in situ hybridisation and immunostaining on Sox1-GFP^{+/-} mouse cerebellum sections. Expression in sagittal sections of early (A,E), mid (B,F) and late (C,G) stages of postnatal cerebellar maturation was compared to adult (D,H) tissue. RNA in situ hybridisation (blue, top panels) shows Sox2 and Sox9 expression becoming progressively restricted to the Purkinje cell layer. Bottom panels show corresponding images obtained from immunostaining for GFP (red). (A,E) P4, (B,F) P7, (C) P25, (G) P15, (D,H) adult. ML: molecular layer, WM: white matter, IGL: internal granule layer. Bar=50 µm.

Figure 4: Analysis of GFP-positive cells in disaggregated brain tissue from adult Sox1-GFP^{+/-} mouse. (A-B) FACS analysis of cells isolated from the ventricular area (A) showing a large GFP⁺ fraction, and comparison with cell suspension prepared from cerebellar cortex (B) highlighting the presence of a GFP⁺

population. Control samples (black line) were collected from wild type littermates. (C-F) Formation of neurosphere-forming cells from mature Sox1-GFP^{+/+} cerebellar tissue observed after 2 (C,D) and 9 (E,F) days in culture. GFP signal was visible under fluorescence (C,E, arrow) in primary cultures. (D,F) Corresponding phase contrast view. (G) CB-NLC cultures after the first passage. (H) CB-NLCs grow as a monolayer when seeded on gelatine, displaying radial glia morphology (insert). Bar=100 μ m.

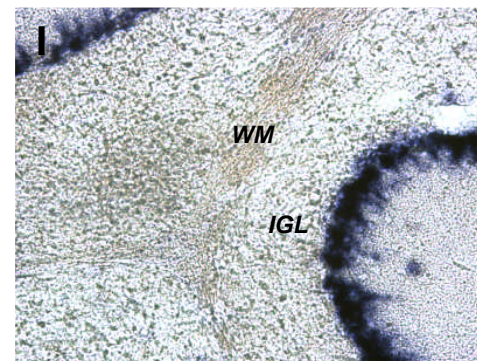
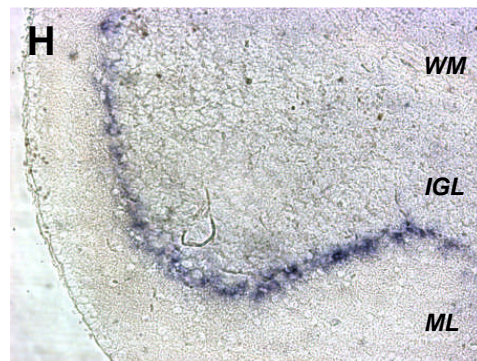
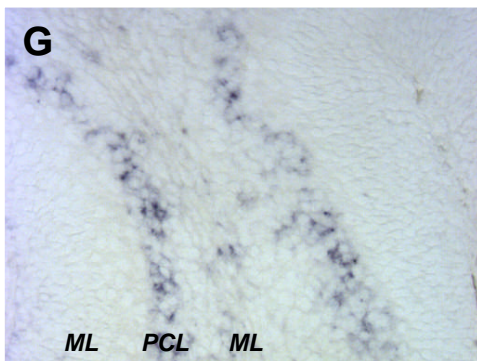
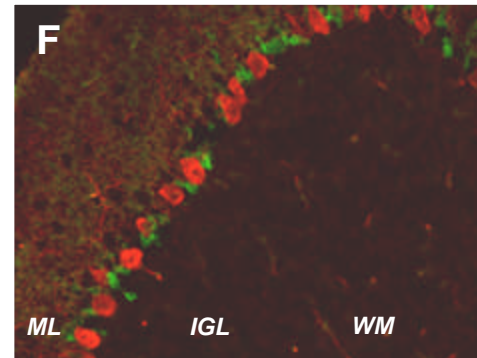
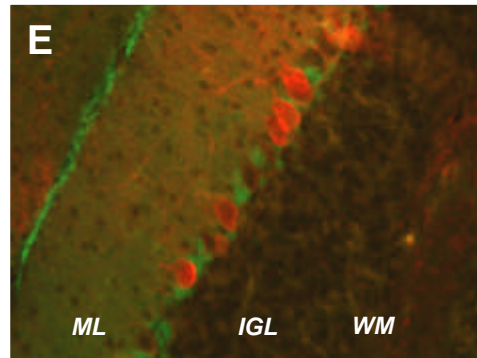
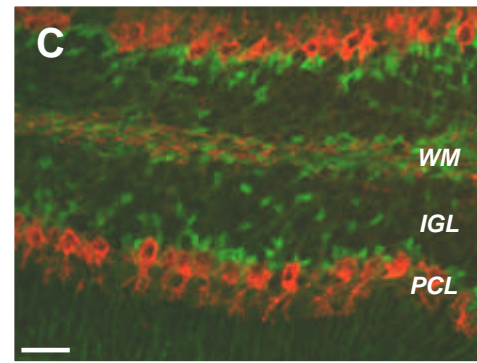
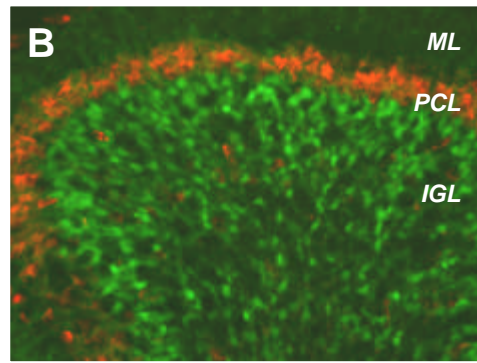
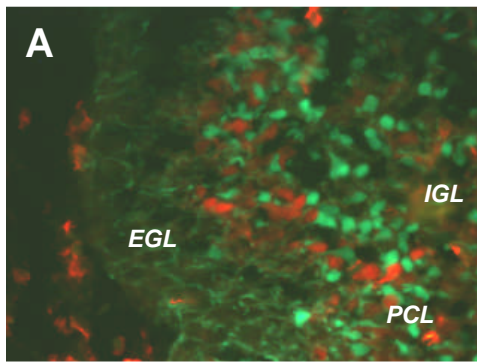
Figure 5: Characterisation of the stock CB-NLC culture prepared from adult cerebellar tissue. (A) FACS analysis of Nestin, cd133 and Sox2 expression in CB-NLCs (red) compared to control NSCs (blue) and no primary control (black). (B) Immunostaining characterisation of CB-NLC cells, showing expression of A2B5, Isl1, Vimentin, cd133, BLBP and Nestin. Bottom panels show immunodetection of Sox2 and Sox9 with corresponding dapi counterstain highlighting nuclear staining (arrows). (C) RT-PCR analysis of CB-derived neurosphere-forming cells (CB) and neural stem cells from ventricular tissue (NSC). Expression of transcripts from intronless sequences (left panel) analysed in comparison to -RT control shows expression of Sox1, Sox2 and the GFP reporter, whereas Sox3 was not detected. Cross intron pcr for a range of neural markers (right panel) show similar expression pattern between CB-NLCs and control NSCs from the ventricular zone. Neither population exhibited Oct4 expression. Bar=50 μ m.

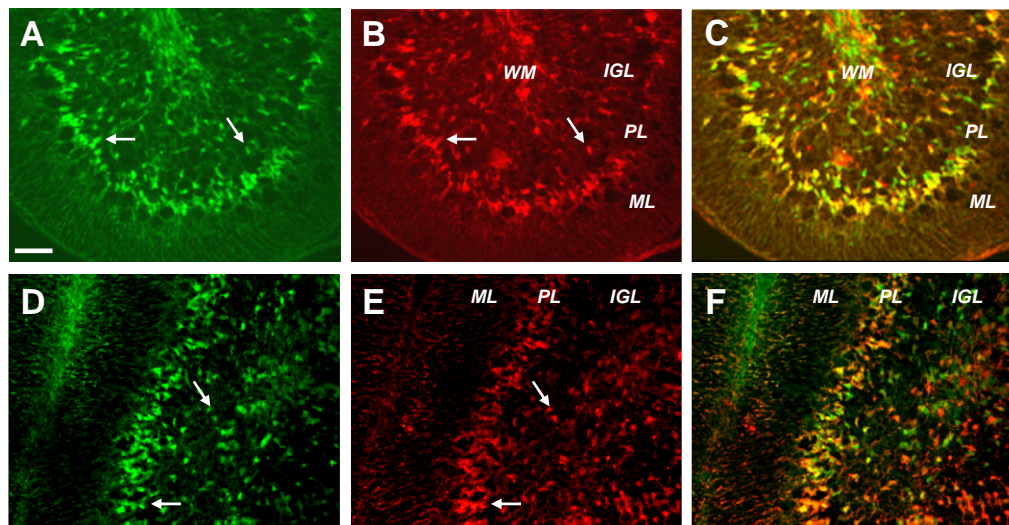
Figure 6: Cellular analysis of a clonal CB-NLC culture. (A) Principle of single-cell cloning and expansion. (B,D) Immunostaining on CB-NLC clonal culture in normal culture conditions (B) and after differentiation (D). (B) Undifferentiated cells stained for nestin (green) and Sox2 (red) with corresponding dapi counterstain and Nestin/Sox2 overlay. (C) FACS analysis of undifferentiated CB-NLC clonal culture showing positive Nestin, Sox2, cd133 and ABCG2 staining compared to no primary control (black). (D) Differentiated cultures stained for CNPase, GFAP, β 3-Tubulin and NF200 with dapi counterstain (blue). Bar=50 μ m.

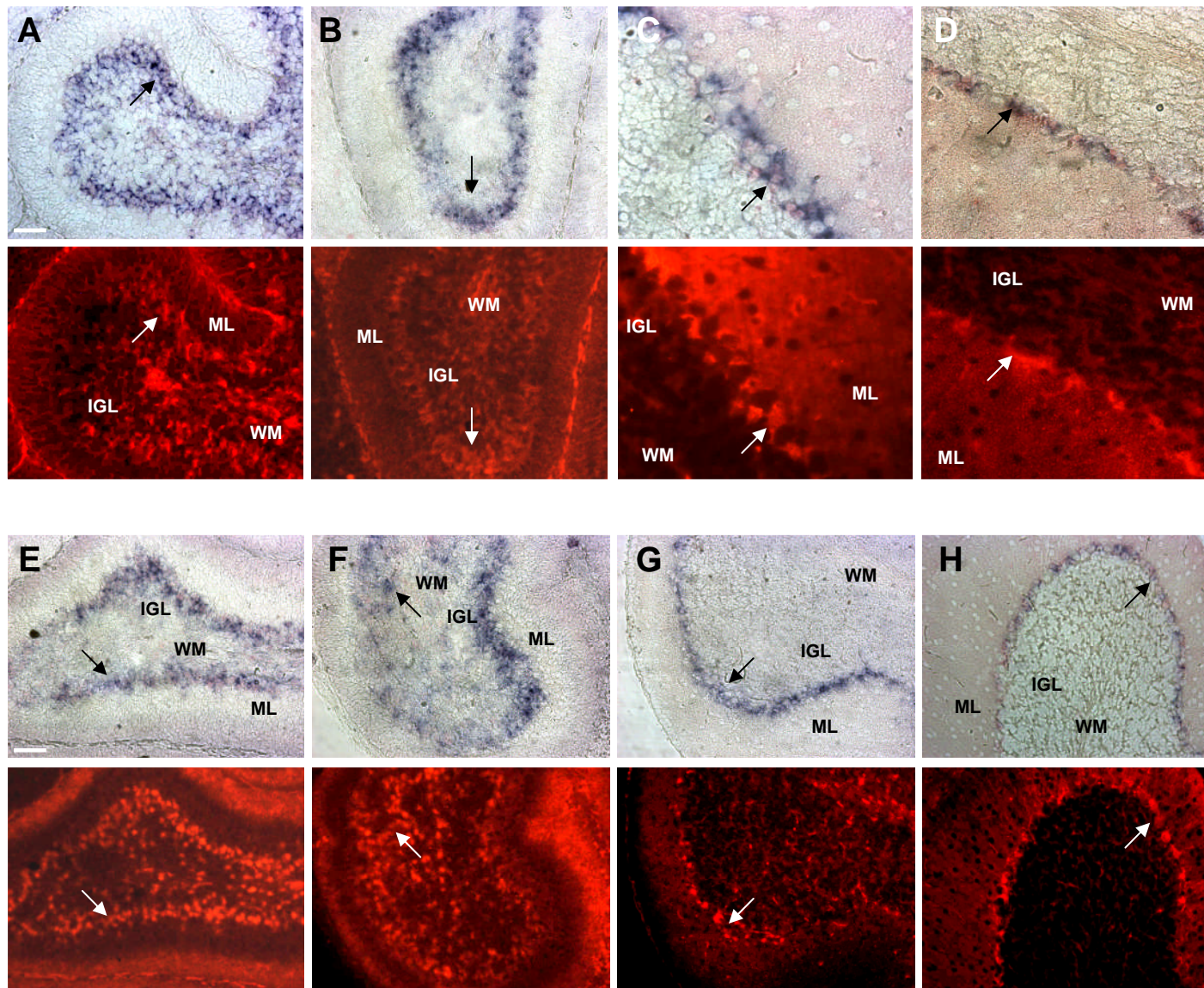
Supplementary Figure 1: Gene expression analysed on sagittal sections of chick cerebellum (HH stages 40-44). (A-B) Immunostaining for Sox1 shows strong signal (brown) in the Purkinje cell layer, with corresponding dapi counterstain (B). (C-D) Analysis of Sox2 (C) and Sox9 (D) expression by RNA in situ hybridisation (blue) highlights the Purkinje cell layer (marked by hatched line). Expression is detected in

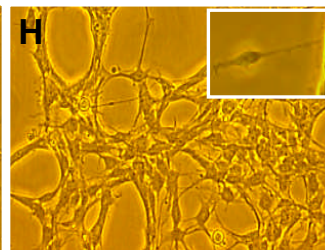
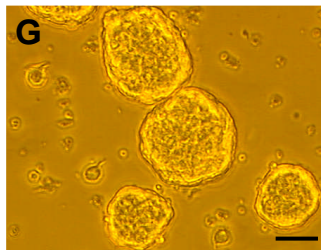
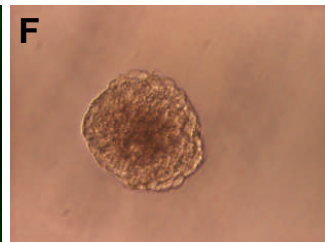
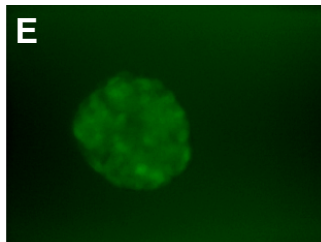
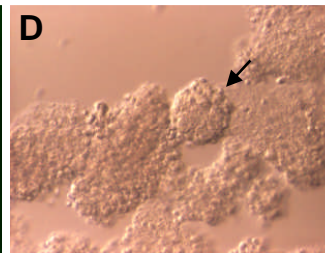
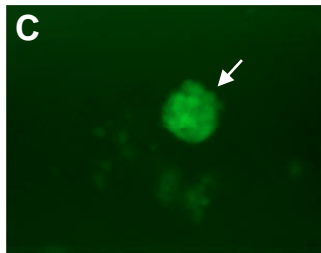
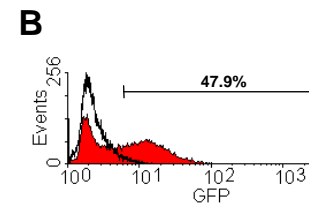
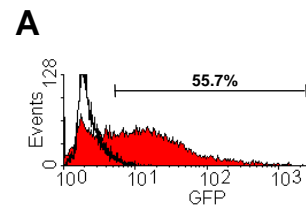
immature Bergmann Glia which are migrating to the Purkinje cell layer (shown between dashed lines), highlighted by calbindin-positive immunostaining (brown). At these stages of development, expression of Sox1, Sox2 and Sox9 is also seen in the white matter and occasionally the inner granular layer (C,D). PCL: Purkinje cell layer; WM, White Matter; IGL, Inner Granular Layer; ML, Molecular Layer. Bar=50 μ m.

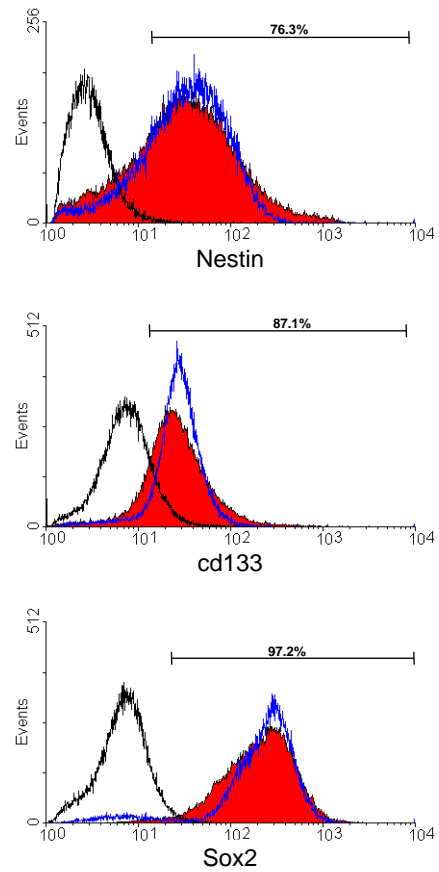
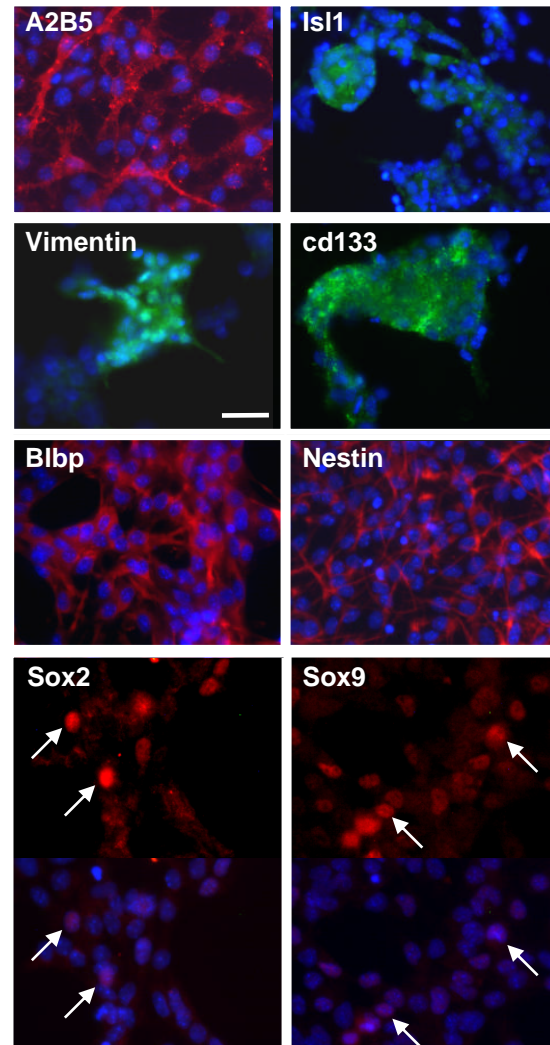
Supplementary Figure 2: FACS analysis of P7 cerebellum from Sox1-GFP^{+/-} mouse, showing that 90% of cd133-labelled cells are found to be GFP-positive (red histogram), compared to cd133-labelled cells from wt cerebellum control (black line).









A**B****C**