© 2017. Published by The Company of Biologists Ltd | Development (2017) 144, 3388 doi:10.1242/dev.158600

CORRECTION

Correction: Distinct roles of neuroepithelial-like and radial glia-like progenitor cells in cerebellar regeneration

Jan Kaslin, Volker Kroehne, Julia Ganz, Stefan Hans and Michael Brand

There was an error in Development **144**, 1462-1471.

Michael Brand should have been listed as co-author for correspondence. Correct details for correspondence are shown below:

Authors for correspondence (jan.kaslin@monash.edu; michael.brand@biotec.tu-dresden.de)

The online article has been amended. The authors apologise to readers for this mistake.

© 2017. Published by The Company of Biologists Ltd | Development (2017) 144, 1462-1471 doi:10.1242/dev.144907

STEM CELLS AND REGENERATION

RESEARCH ARTICLE

Distinct roles of neuroepithelial-like and radial glia-like progenitor cells in cerebellar regeneration

Jan Kaslin[1,2,‡], Volker Kroehne[1], Julia Ganz[1,*], Stefan Hans[1] and Michael Brand[1]

ABSTRACT

Zebrafish can regenerate after brain injury, and the regenerative process is driven by resident stem cells. Stem cells are heterogeneous in the vertebrate brain, but the significance of having heterogeneous stem cells in regeneration is not understood. Limited availability of specific stem cells might impair the regeneration of particular cell lineages. We studied regeneration of the adult zebrafish cerebellum, which contains two major stem and progenitor cell types: ventricular zone and neuroepithelial cells. Using conditional lineage tracing we demonstrate that cerebellar regeneration depends on the availability of specific stem cells. Radial glia-like cells are thought to be the predominant stem cell type in homeostasis and after injury. However, we find that radial glia-like cells play a minor role in adult cerebellar neurogenesis and in recovery after injury. Instead, we find that neuroepithelial cells are the predominant stem cell type supporting cerebellar regeneration after injury. Zebrafish are able to regenerate many, but not all, cell types in the cerebellum, which emphasizes the need to understand the contribution of different adult neural stem and progenitor cell subtypes in the vertebrate central nervous system.

KEY WORDS: Zebrafish, Cerebellum, Neural stem cell, Neurogenesis, Radial glia, Regeneration

INTRODUCTION

Neural stem and progenitor cell populations are heterogeneous and biased in their ability to produce specific cell types in the vertebrate brain (Shen et al., 2006; Merkle et al., 2007; Kaslin et al., 2009; Ganz et al., 2010; Marz et al., 2010). The implication of heterogeneous brain stem cells for tissue regeneration is not well understood. However, the limited availability or depletion of specific stem and progenitor cells could impair the regeneration of specific brain cell lineages (Liu et al., 2009; Encinas et al., 2011; Barbosa et al., 2015). Teleost fish have a much greater ability to regenerate CNS injuries than mammals (Becker et al., 1997; Kaslin et al., 2008; Reimer et al., 2008; Kroehne et al., 2011; Baumgart et al., 2012; Kishimoto et al., 2012; Kizil et al., 2012). Neural regeneration in the zebrafish telencephalon is driven by distinct tissue-resident stem and progenitor cells (Reimer et al., 2008; Kroehne et al., 2011; Barbosa et al., 2015), but cellular regeneration has not been studied in detail in other brain parts. Zebrafish maintain widespread neural stem cell activity along the neural axis throughout life, suggesting that stem cells could potentially repair injury in any brain region (Adolf et al., 2006; Grandel et al., 2006; Kaslin et al., 2008). The current consensus drawn from relatively few studies in one brain region, the telencephalon, is that the teleost brain can repair most, if not all, cell types (Reimer et al., 2008; Kroehne et al., 2011; Barbosa et al., 2015). However, it is unclear whether all cell types can be regenerated after brain injury in every part of the brain.

We previously demonstrated that the zebrafish cerebellum contains stem and progenitor cells with different capacities to generate neurons and glia over their life span (Kaslin et al., 2009, 2013). Two main types of stem and progenitor cells give rise to the different cells in the juvenile and adult cerebellum. Neuroepithelial-like stem and progenitor cells in the upper rhombic lip continuously give rise to granule cells in adult and aging zebrafish (Kaslin et al., 2009, 2013). Progenitor cells in the ventricular zone (VZ) give rise to the other cell lineages, such as Purkinje cells, Golgi, stellate and eurydendroid cells, and VZ progenitor cell activity and production nearly ceases in the adult zebrafish cerebellum (Kaslin et al., 2009, 2013). Given these differences in stem and progenitor cell behaviour we sought to determine which stem and progenitor cell population(s) respond to injury and contribute to cellular regeneration in the zebrafish cerebellum. The zebrafish cerebellum is ideally suited for this because it has well-defined cellular architecture and cell types (Bae et al., 2009; Kaslin et al., 2009; Kaslin and Brand, 2012) that have direct counterparts in the mammalian cerebellum (Kaslin and Brand, 2012).

We performed a unilateral surgical injury that removed all cerebellar cell types in order to determine the capacity of adult zebrafish to regenerate these diverse cell types. By histological analysis, BrdU pulse chases and genetic lineage tracing we found that only specific cell lineages regenerate well after injury in the adult cerebellum. To confirm the role of different stem and progenitor cell populations in recovery we used stem cell-specific reporters and proliferation markers. Stem and progenitor cell activity is significantly upregulated after injury and the capacity to generate specific cell lineages directly correlates with the presence of particular stem and progenitor cell types in the adult cerebellum. We found that the population of neuroepithelial-like stem cells declined in the juvenile fish but remained stable in the aging cerebellum. Contrary to the behaviour of the neuroepithelial-like stem cells, the number and activity of VZ progenitor cells was nearly exhausted. However, juvenile fish up to 1 month demonstrate activity of both stem and progenitor cell types and production of all major cerebellar cell types. Injury experiments indicated that the juvenile fish can regenerate all cell lineages. Taken together, our data demonstrate that only cell types that are homeostatically produced in the adult zebrafish cerebellum regenerate well, and that

[1]Biotechnology Center and Center for Regenerative Therapies Dresden, Dresden University of Technology, Tatzberg 47 01307, Germany. [2]Australian Regenerative Medicine Institute, Monash University, Innovation Walk 15, Lvl1, 3800, Clayton, Melbourne, Australia.
*Present address: Department of Integrative Biology, Michigan State University, 288 Farm Lane, East Lansing, MI 48824, USA.

‡Author for correspondence (jan.kaslin@monash.edu)

J.K., 0000-0002-6207-1889

Received 20 September 2016; Accepted 3 March 2017

Development (2017) 144, 1462-1471 doi:10.1242/dev.144907

cerebellar stem and progenitor cells retain their specified cell lineages even after injury. In contrast to the zebrafish telencephalon, radial glia-like cells play only a minor role in the adult cerebellum during homeostasis or after injury. Our findings highlight the importance of understanding the heterogeneity of stem cells *in vivo*. Importantly, our data challenge the predominating view that zebrafish can regenerate all cell types in the CNS.

RESULTS

Morphological and behavioural recovery after cerebellar injury

To address whether all cerebellar cell types can be regenerated after injury we performed a surgical lesion paradigm (‘trepanation’) whereby all cerebellar cell types are removed from the adult zebrafish (≥6 months old; Fig. 1A). The surgery unilaterally

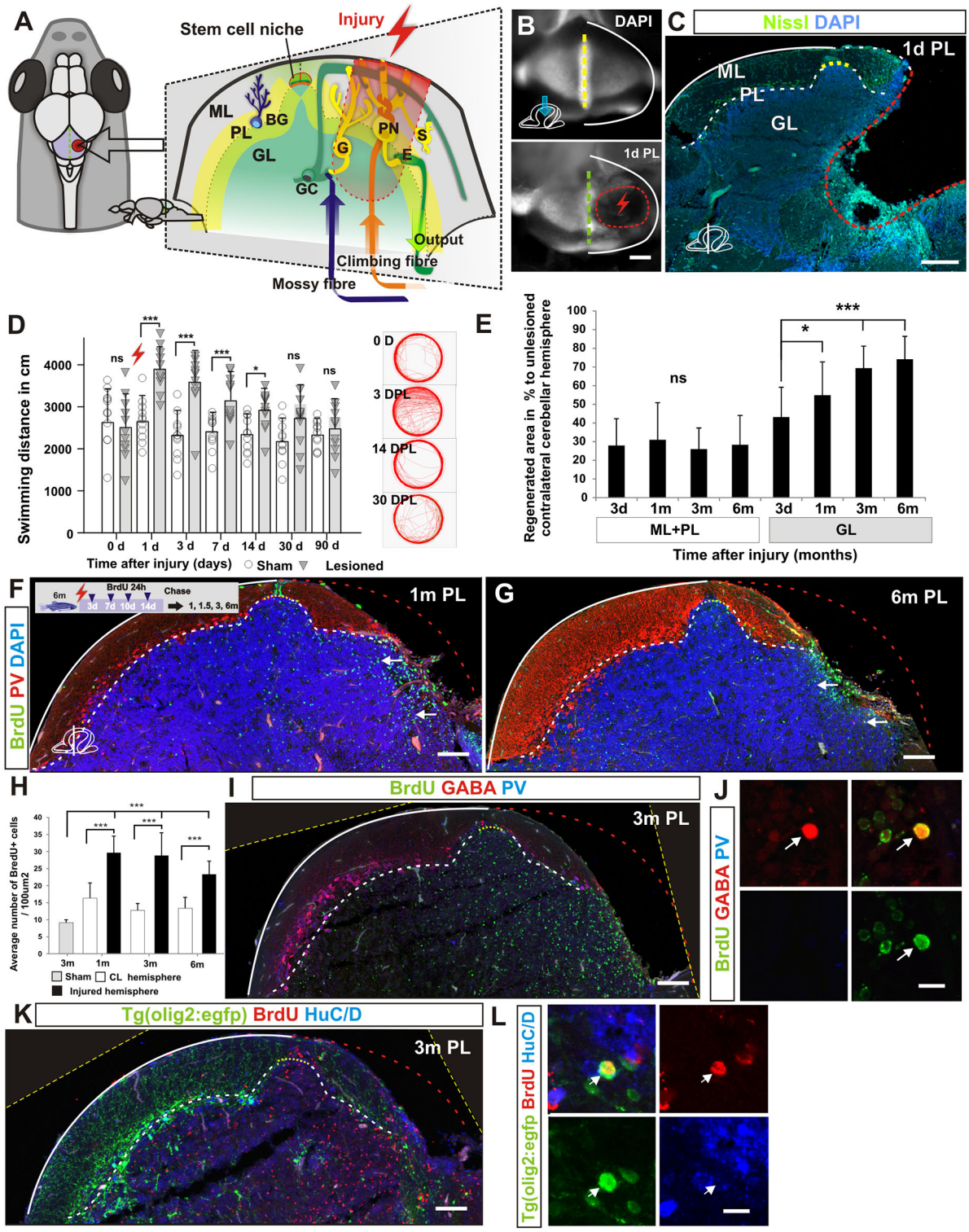

Fig. 1. See next page for legend.

Development (2017) 144, 1462-1471 doi:10.1242/dev.144907

Fig. 1. Morphological and behavioural recovery after cerebellar injury. (A) Overview of cerebellar architecture in zebrafish and the injury model. The cerebellum contains only a few cell types of distinct morphology. The cerebellum has a simple laminar architecture consisting of a molecular layer (ML), Purkinje cell layer (PL) and granule cell layer (GL). The unilateral injury removes cells from all three layers. Importantly, the lesion spares the stem cell niche. The GL consists of densely packed excitatory granule cells (GC) and inhibitory Golgi neurons (G). The PL contains Purkinje neurons (PN), specialised Bergmann glia (BG), and excitatory eurydendroid cells (E). The ML consists of nerve fibres and scattered inhibitory stellate cells (S). Neural stem cells are maintained in the dorsomedial part of the cerebellum around a remnant of the IVth ventricle. (B) A piece of the corpus cerebelli, including all cell layers, is unilaterally removed (red lightning). A confocal maximum projection showing a dorsal view of an unlesioned (top) and a lesioned (bottom) cerebellum 1 day after the injury. The stem cell niche is indicated with a yellow dashed line. The extent of the lesion is indicated with a red dashed line. (C) A transverse section of the corpus cerebelli showing the lesion 1 day after injury. (D) Automatic tracking of swimming distance and pattern during 5 minutes. Cerebellar injury induced significantly increased swimming activity from 1 to 14 days after injury. n=11 for sham and injured groups; 1, 3, 7 days post-lesion (DPL) ***P<0.001, 14 DPL *P<0.05; error bars indicate s.d. (E) Morphological quantification of the lesioned area after injury. Lesioned area compared with lesion size measured 3 days after injury. n≥5 per time point; 1 month *P<0.05, 3 and 6 months ***P<0.001; ns, not significant; error bars indicate s.d. (F,G) Transverse section of the corpus cerebelli showing that BrdU-stained cells (green) are recruited and maintained (arrows) in the GL of the lesioned hemisphere 1 and 6 months after injury. Few BrdU-stained cells are detected in the ML and PL. Purkinje cells are stained with parvalbumin (PV; red). (H) Quantification of BrdU-positive cells in the GL in the injured and contralateral hemisphere 1 , 3 and 6 months after injury. n=5 per time point; ***P<0.001; error bars indicate s.d. Sham control animal is included as reference point for normal granule cell production rate (3 months after BrdU pulse). CL, contralateral uninjured hemisphere. (I,J) Cerebellar transverse section showing BrdU, GABA and PV staining 3 months after injury. Very few BrdU/GABA-positive cells are detected (arrow). (K,L) Cerebellar transverse section showing *olig2:gfp*, BrdU and HuC/D staining 3 months after injury. BrdU/*olig2:gfp*-positive cells are very rarely detected (arrow). Yellow dashed line indicates original picture border in rotated images. Scale bars: 200 µm (B); 150 µm (C); 100 µm (F,G,I,K); 10 µm (J,L).

removed 29±6% of the corpus cerebelli volume (n=7, measured 1 day after injury; Fig. 1B,C). Importantly, the lesion spared the cerebellar recessus and the associated stem cell niche (Fig. 1B,C, Fig. S1A) (Kaslin et al., 2009, 2013). Rapid wound healing, with regeneration of skin and bone, was detected within days after the lesion (Fig. S1B). During the first weeks following injury, postural and locomotory behavioural deficits characterised by erratic rapid swimming were detected in the injured fish, but these deficits gradually disappeared within 4 weeks (Fig. 1D; P<0.001 for 1, 3 and 7 days post injury, P<0.05 for 14 days post injury, n=11 each for sham-injured and injured groups).

To analyse if the functional recovery was accompanied by morphological regeneration we quantified tissue recovery 1, 3 and 6 months after injury (Fig. 1E). Morphological quantifications of lesion size showed that the granule cell layer area increased significantly 1 month after injury and very significantly 3 to 6 months after the lesion, as compared with 3 days after injury (Fig. 1E; n≥5 per time point, 1 month P<0.05, 3 and 6 months P<0.001). By contrast, the Purkinje cell layer and the molecular layer were not significantly increased after injury (Fig. 1E; n≥5 per time point, 1 month P=0.835, 3 months P=0.678, 6 months P=0.754). The Purkinje cell layer and the molecular layer remained noticeably perturbed 12 months after injury, indicating that the cell types in these layers regenerated poorly (n=5; Fig. S1C). However, we detected restoration of the granule cell layer.

Cellular recovery after cerebellar injury

The recovery of the tissue could be caused by general tissue movements and cell migration. To determine if cells are produced after injury we performed a series of BrdU pulse-chase experiments in combination with immunohistochemical stainings for cell type-specific markers. To detect cells that were proliferating after injury we performed consecutive 24-h continuous pulses of BrdU given on day 3, 7, 10 and 14 after the injury (Fig. 1F). In agreement with the morphological analysis, the BrdU pulse-chase experiments showed that the vast majority of cells produced after injury were located in the granule cell layer (Fig. 1F,G). Newly produced cells might not survive for any extensive period in the adult brain, and this is particularly noticeable after injury (Kernie and Parent, 2010; Magnusson et al., 2014). However, in agreement with the morphological analysis, the number of granule cells produced *de novo* more than doubled and these were maintained at the lesioned hemisphere 3 to 6 months after the lesion, as compared with the unlesioned hemisphere or sham control (n≥5 per time point, P<0.001; Fig. 1H). No difference in the number of BrdU-labelled cells was detected between 3 and 6 months, suggesting that the cells that were produced were stably maintained over time (n≥5 per time point, P>0.05; Fig. 1H).

To determine if inhibitory cell lineages were produced we used γ-aminobutyric acid (GABA) and parvalbumin (PV), Zebrin II (ZII; Aldoca – ZFIN) and Pax2 to further identify Purkinje and Golgi neurons. GABA was used to identify all inhibitory neurons, while PV and ZII were used to label Purkinje cells (Bae et al., 2009; Kaslin et al., 2013). Pax2 is required for the differentiation of Golgi and stellate cells in the mouse cerebellum (Maricich and Herrup, 1999; Kaslin et al., 2013). In agreement with the morphological analysis, only a very small number of GABA/BrdU-immunopositive inhibitory neurons were detected 3 months after injury (n=5, 16.74±7.33 cells/brain; Fig. 1I,J, Fig. S1E). A similar number of Pax2/BrdU-immunopositive cells were detected 3 months after injury, suggesting that primarily Golgi cells were produced (n=5, 11.23±9.67 cells/brain; Fig. S1D). No BrdU-labelled PV^+ or ZII^+ Purkinje neurons were detected at any of the analysed time points after injury (n=5 for each time point, Purkinje cell layer at 1.5, 3 and 6 months; Fig. 1F-J, Fig. S1). Eurydendroid cells were identified using the *olig2:egfp* transgenic line that labels most eurydendroid cells and oligodendrocytes in the corpus cerebelli (Bae et al., 2009; Kaslin et al., 2013). No BrdU-labelled *olig2:egfp*-positive cells (eurydendroid cells) were detected 3 months after injury (n=5; Fig. 1K), but rare BrdU and *olig2:egfp* double-positive and neuronal marker-negative cells (oligodendrocytes) were observed in the granule cell layer (n=5, 5.3±2.6 cells/brain; Fig. 1L). Glutamine synthetase (GS) or S100β labelling was used to identify Bergmann glia (Kaslin et al., 2009, 2013). A small number of BrdU and GS/S100β double-positive glial cells were detected 3 months after injury (n=5, 24.6±6.4 cells/brain; data not shown; see Fig. 3C-E). Furthermore, we performed unilateral needle stick injury to restrict damage, since injury size could hamper the regenerative response. In agreement with the surgical injury, no BrdU-labelled PV^+ or ZII^+ Purkinje neurons were detected 4 weeks after injury (n=5; data not shown). Taken together, our data demonstrate that granule cells readily regenerate after injury, but regeneration of other cell types is limited in the adult cerebellum.

Activation of neural stem and progenitor cells after cerebellar injury

Neural regeneration in the zebrafish brain is driven by distinct tissue-resident stem and progenitor cells (Kroehne et al., 2011;

Development (2017) 144, 1462-1471 doi:10.1242/dev.144907

Barbosa et al., 2015). However, it is known that stem and progenitor cells are heterogeneous in the mammalian and zebrafish brain (Merkle et al., 2007; Kaslin et al., 2009; Ganz et al., 2010; Marz et al., 2010). The significance of heterogeneous stem cell populations in tissue regeneration remains unclear. Conceivably, limited availability or depletion of specific stem cells could impair the regeneration of specific cell lineages. In the cerebellum, two distinct populations of neural stem and progenitor cells are established early during development, but only one of the populations remains active in the adult (Kaslin et al., 2009, 2013). The maintained and actively cycling stem cell population displays neuroepithelial-like characteristics and primarily produces granule neurons (Kaslin et al., 2009, 2013). The other population with stem cell and progenitor cell characteristics is located at the VZ, displays radial glia-like characteristics, and produces inhibitory neuronal cell lineages and glia in the juvenile cerebellum. This population gradually becomes quiescent or, alternatively, is exhausted in the adult coincident with the ceased production of most inhibitory cell lineages in the cerebellum (Kaslin et al., 2013). Exhaustion of neural stem and progenitor cells over time might be a general trait of vertebrate brains, since recent studies have highlighted the possibility that the neural stem pool is gradually depleted over time in the zebrafish and rodent brain (Barbosa et al., 2015). We sought to determine which cell types respond to injury and contribute to cellular regeneration.

The transgenes *ptf1a:DsRed* and *nestin:gfp* distinctly label VZ progenitor cells and neuroepithelial-like progenitor cells, respectively, in the zebrafish cerebellum, and they can be used to monitor stem and progenitor cell activity (Kani et al., 2010; Kaslin et al., 2013). Furthermore, Ptf1a is required for the generation of all inhibitory neuronal lineages in the cerebellum (Hoshino et al., 2005). To determine if stem and progenitor cell activity declines with age, we quantified the number of *nestin:gfp*$^{+}$ and *ptf1a:DsRed*$^{+}$ cells in the young and aging zebrafish cerebellum (3, 6, 14 and 22 months). The number of *nestin:gfp*$^{+}$ neuroepithelial-like cells very significantly declined over time in the aging cerebellum (Fig. 2A; $n \geq 5$ per time point, $P<0.001$). However, no significant decline in cell number was detected between 14 and 22 months,

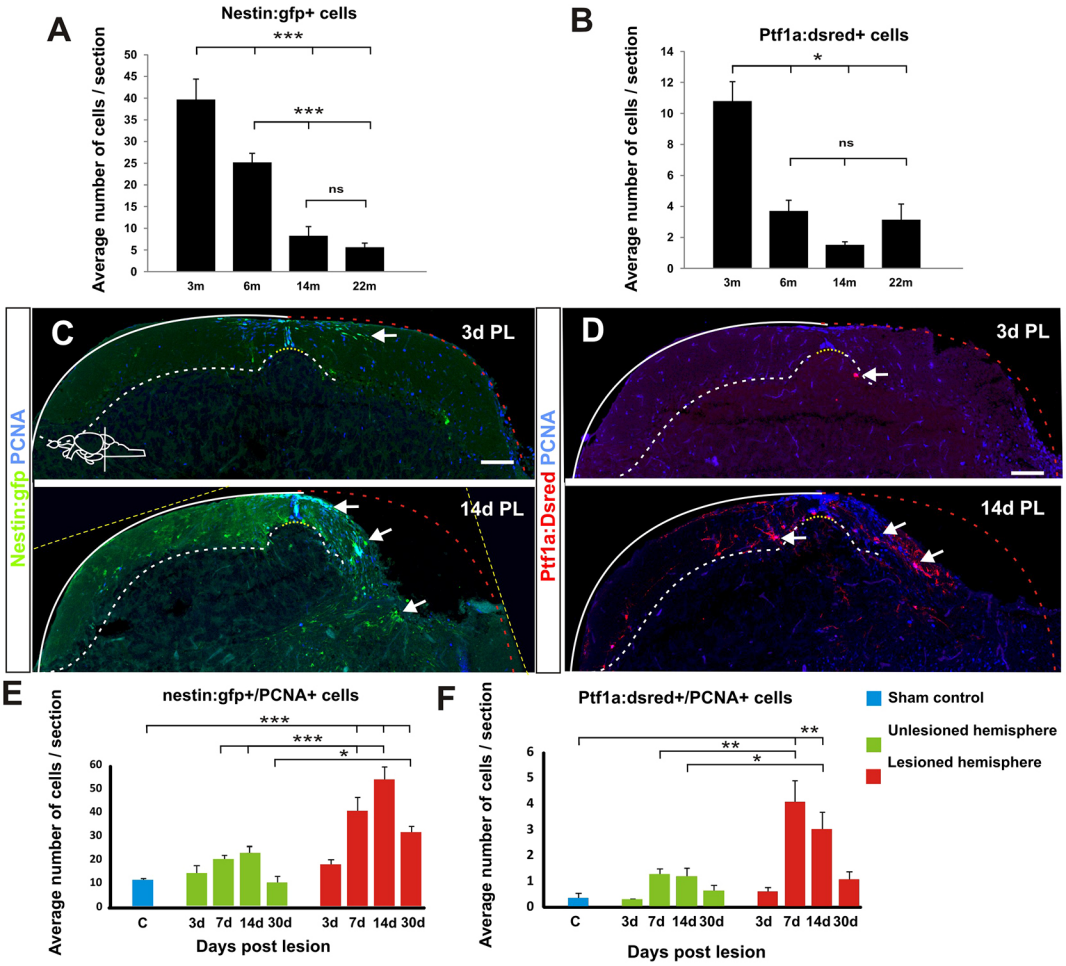

Fig. 2. Stem and progenitor cell response to cerebellar injury. (A,B) Quantification of *nestin:gfp* and *ptf1a:DsRed* cells in the aging cerebellum during homeostasis. *nestin:gfp*: n=5 at 3, 6 and 22 months, n=6 at 14 months, ***P<0.001; *ptf1a:DsRed*: n=7 at 3, 6 months, n=6 at 14 months, n=5 at 22 months, *P<0.05; error bars indicate s.e.m. (C,D) Maximum projections of cerebellar transverse sections showing stem cell activation after injury. Initially, the tissue morphology is severely altered at the level of the injury and therefore sections from the very rostral end of the lesion were chosen to show the stem cell response. Three days after the lesion, both *ptf1a:DsRed*$^{+}$ (red) and *nestin:gfp*$^{+}$ (green) stem cell activity (arrows) is unaltered. Stem cell activity (arrows) is significantly increased 7 days after injury and peaks ∼2 weeks after injury. Proliferating cells are labelled with Pcna (blue). (E,F) Quantification of cerebellar stem cell activity after injury. A significant increase in the number of *nestin:gfp*$^{+}$ cells is seen from 7 to 30 days after the lesion. Sham control n=5, 3 DPL n=4, 7 DPL n=6, 14 DPL n=5, 30 DPL n=5; *P<0.05, ***P<0.001; error bars indicate s.e.m. A significant increase in *ptf1a:DsRed*$^{+}$ stem cells is detected 7 and 14 days after injury. Sham control n=7, 3 DPL n=4, 7 DPL n=5, 14 DPL n=5, 30 DPL n=5; *P<0.05, **P<0.01; error bars indicate s.e.m. Note the differences in scale on the y-axis between E and F (60 versus 6 cells). Yellow dashed line shows original picture border in rotated images. Scale bars: 100 µm.

Development (2017) 144, 1462-1471 doi:10.1242/dev.144907

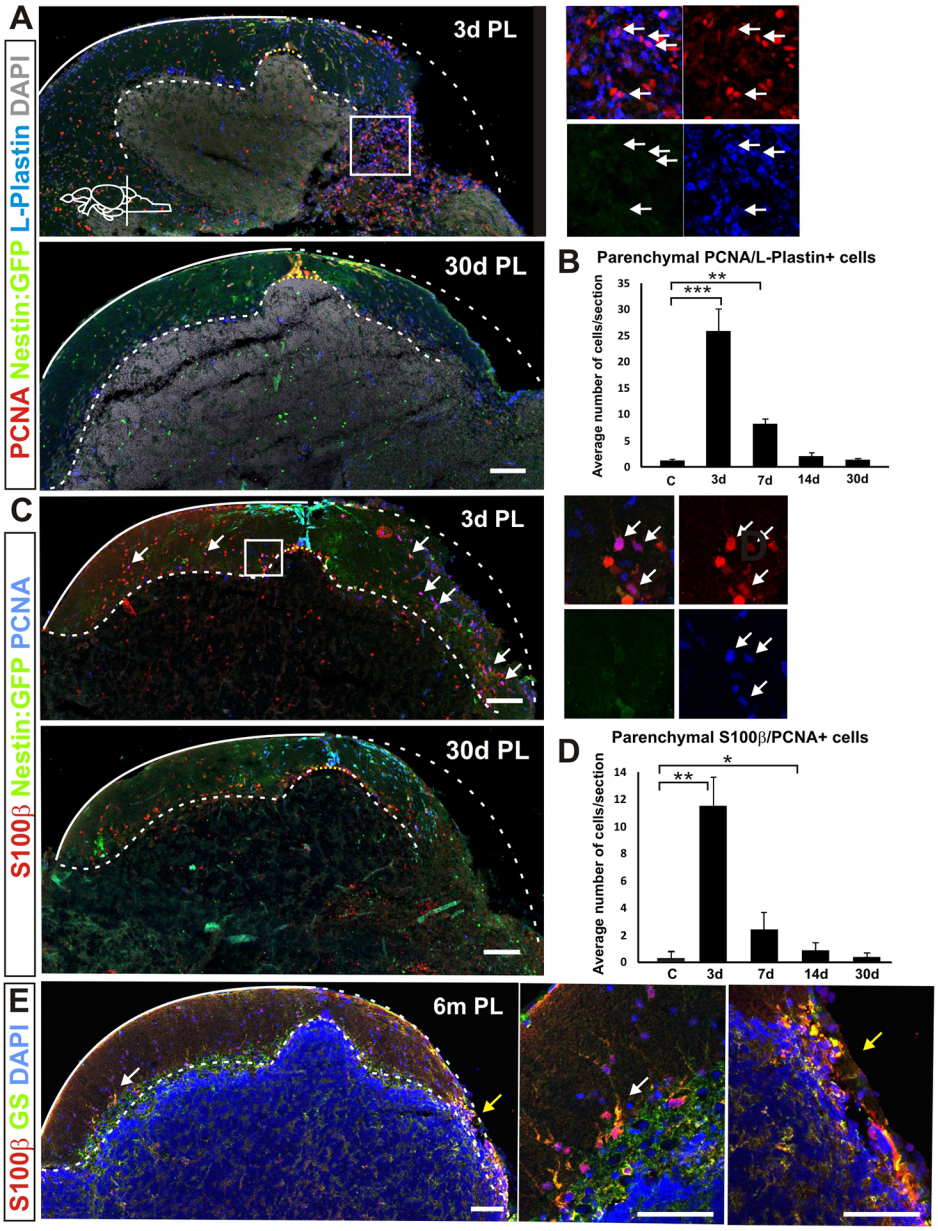

Fig. 3. Inflammation and scarring after injury. (A) Cerebellar transverse section showing proliferating (Pcna$^+$) *nestin:gfp*$^-$ parenchymal cells (green) colocalising with the leukocyte marker L-plastin (arrows) 3 days after injury. Parenchymal proliferation and leukocyte numbers were restored towards homeostatic levels 30 days after injury. (B) Quantification of proliferating Pcna/L-plastin-positive cells in the cerebellar parenchyma after injury. A significant increase in the number of proliferating L-plastin$^+$ cells is detected during the first week. The number of proliferating L-plastin$^+$ cells is back to control levels 14 days after injury. $^{**}P$<0.01, $^{***}P$<0.001; n=5 for each time point. (C) Proliferating Bergmann glia (Pcna/S100β$^+$) are also detected in the parenchyma after injury (arrows). (D) Quantification of proliferating Bergmann glia shows a significant number in the cerebellar parenchyma 3 days after injury. $^*P$<0.05, $^{**}P$<0.01; n=55 for each time point. The number of proliferating Bergmann glia decreases the first week after injury and is back to homeostatic levels 30 days after injury. (E) Glial proliferation does not result in glial scarring. No notable accumulation of the glial markers S100β (red) and GS (green) can be detected at the injury site 6 months after injury (n=5). The arrowed regions are magnified to the right, illustrating proliferating leukocytes in A and proliferating Bergmann glia in C (white arrows) and Bergmann glia at the lesion site (yellow arrows). Scale bars: 100 µm.

suggesting that the active cerebellar stem cell pool remains stable in the aging cerebellum (Fig. 2A). The number of *ptf1a:DsRed*$^+$ cells significantly declined in the aging cerebellum and was very low in the adult (Fig. 2B; $n\geq5$ for each time point, P<0.05). However, no difference in *ptf1a:DsRed*$^+$ cell number was detected between 6 months and later time points, suggesting that the population remains stable and largely quiescent from 6 months onwards (Fig. 2B).

To determine if stem and progenitor cells can be activated after injury, we performed a quantitative colocalisation analysis with the cell proliferation marker Proliferating cell nuclear antigen (Pcna) in the lesioned cerebellum of 6-month-old *ptf1a:DsRed*$^+$ and *nestin:gfp*$^+$ fish. Pcna is expressed during G1 to G2 phase of the cell cycle and is thus a broad cell proliferation marker. The number of *ptf1a:DsRed /pcna*$^+$ and *nestin:gfp/pcna*$^+$ cells significantly increased during the first 2 weeks following lesion (P<0.01; Fig. 2C-F). A fivefold and elevenfold increase in the number of proliferating *ptf1a:DsRed*$^+$ and *nestin:gfp*$^+$ stem cells was seen in lesioned hemispheres between 7 days and 14 days post lesion (Fig. 2C-F, Fig. S2A,B). The number of proliferating *ptf1a:DsRed /pcna*$^+$ cells returned to uninjured control levels 30 days after lesion (Fig. 2F). We previously showed that the majority of *DsRed*$^+$ cells are non-cycling in the adult cerebellum, and they display morphological and molecular characteristics of differentiating neurons or glia (Kaslin et al., 2013). In agreement with this, the proportion of persisting *DsRed*$^+$, but non-cycling (Pcna$^-$), cells increased 7 days after injury, suggesting that the *DsRed*$^+$ cells exited the cell cycle and are differentiating (Fig. S2C). The number of proliferating *nestin:gfp*$^+$ stem cells was still elevated compared with the uninjured control 30 days after the lesion, showing that neuroepithelial-like stem cell activity remains augmented long after injury (Fig. 2C, Fig. S2A). Taken together, our data demonstrate that both neuroepithelial-like cells and VZ progenitors are activated and proliferate following injury. However, the relative number of activated VZ progenitors is very low even at the peak of activation (<5 cells per section; Fig. 2F). These findings are in agreement with the BrdU pulse-chase experiments, which demonstrated that very few inhibitory neurons are produced (Fig. 1).

Development (2017) 144, 1462-1471 doi:10.1242/dev.144907

Limited long-term scarring and inflammation after cerebellar injury

In addition to the *ptf1a:DsRed*$^{+}$ or *nestin:gfp*$^{+}$ cells at the lesion site, there was an increase in proliferating cells in the cerebellar parenchyma. In agreement with previous studies (Kroehne et al., 2011; Kyritsis et al., 2012), the majority of proliferating parenchymal cells colabelled with the leukocyte marker L-plastin, which labels macrophages and microglia (Fig. 3A,B). A small subpopulation of proliferating S100β^{+} Bergmann glia was also detected in the cerebellar parenchyma (Fig. 3C,D). Proliferating leukocytes and glia are prerequisites for glial scarring and chronic inflammation, known key factors that impair neural regeneration in mammals (Fitch and Silver, 2008). The number of proliferating S100β^{+} and L-plastin^{+} cells in the parenchyma returned to uninjured levels 30 days after the lesion (Fig. 3A,C). There was no accumulation of the glial proteins GS and S100β^{+} 6 months after lesion (Fig. 3E). Furthermore, we could not detect signs of fibrotic scarring using Acid Fuchsin Orange G or Hematoxylin and Eosin

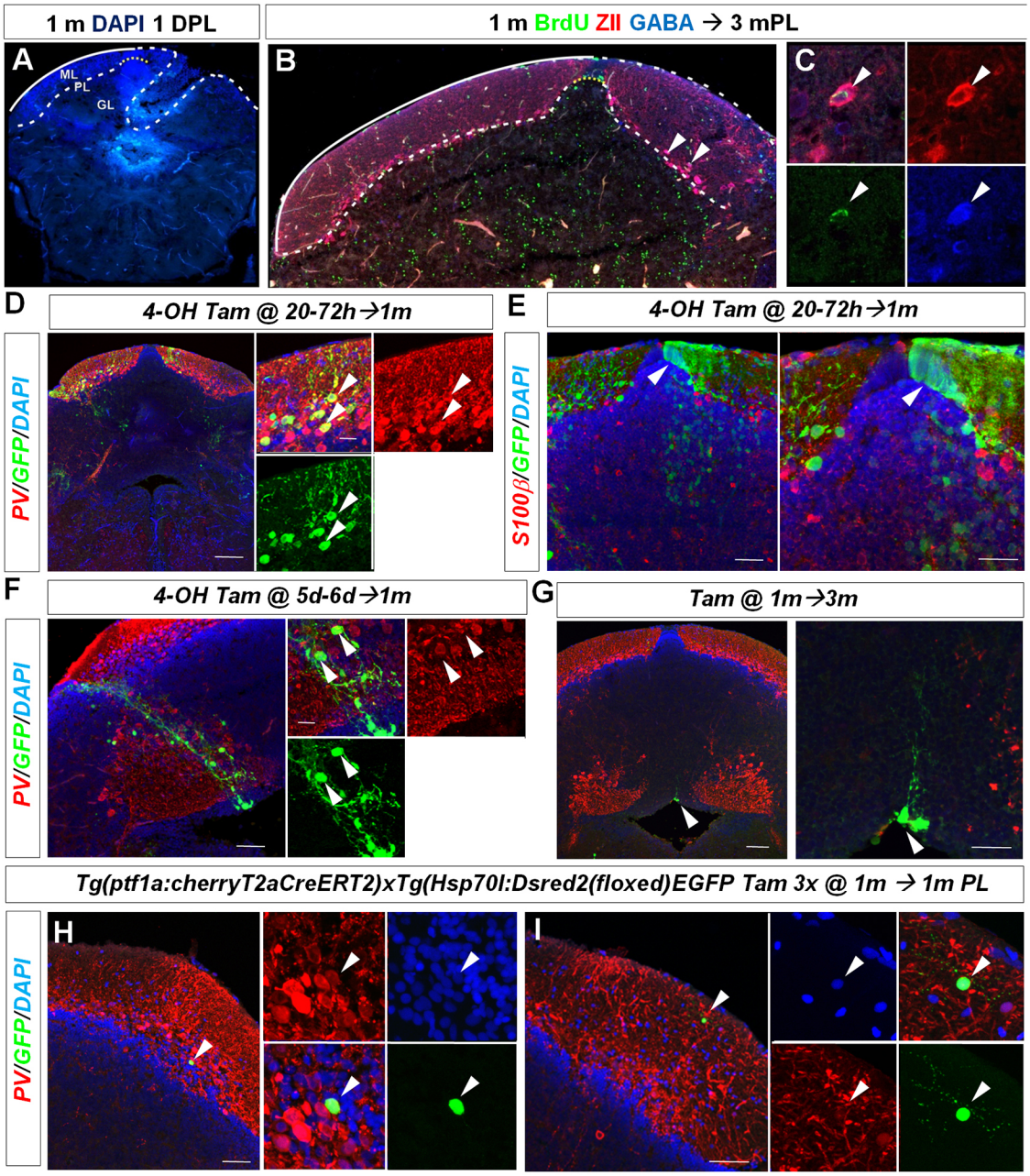

Fig. 4. Juvenile zebrafish regenerate diverse cerebellar cell types. (A) Representative cerebellar transverse section in a juvenile 1-month-old zebrafish showing the lesion area 1 day after injury. (B,C) BrdU/ZII/GABA^{+} Purkinje neurons are found at the lesion site 3 months after injury (arrowheads). (D) Cerebellar transverse section showing recombined GFP/PV^{+} Purkinje cells (arrowheads) in *Tg(ptf1a:cherryT2aCreERT2)*[1]*; Tg(hsp70l:DsRed2(floxed)EGFP)* fish 1 month after recombination. (E) Cerebellar transverse section showing unilateral GFP labelling of neuroepithelial-like stem cells (arrowhead) and granule cells. (F) GFP-labelled radial glia-like cells and a clone of PV^{-} interneurons (arrowheads) recombined between 5 and 6 days postfertilisation. (G) Quiescent GFP-labelled radial glia-like cells (arrowhead) recombined at 1 month and analysed 3 months later. (H) Recombined GFP/PV^{+} Purkinje cell (arrowhead) 1 month after tamoxifen treatment and injury of a juvenile zebrafish. (I) A recombined GFP^{+} and PV^{-} stellate cell (arrowhead) 1 month after tamoxifen treatment and injury. Scale bars: 75 µm (D); 35 µm (E); 50 µm (F); 100 µm (G); 50 µm (H,I).

Development (2017) 144, 1462-1471 doi:10.1242/dev.144907

staining 6 and 12 months following the lesion (Fig. S1C; data not shown). The resolution of inflammation and the lack of any significant glial or fibrotic scarring during recovery are in line with previous findings in zebrafish brain and retina (Kroehne et al., 2011; Baumgart et al., 2012; Kishimoto et al., 2012; Kyritsis et al., 2012).

Juvenile zebrafish can regenerate diverse cerebellar cell types

Our data suggested that there might be a lack of specific stem or progenitor cells in the adult cerebellum to replace specific lineages, such as the Purkinje cells. We previously found that both neuroepithelial and VZ progenitor cells are active in the juvenile zebrafish cerebellum and have the capacity to produce all cell lineages, including Purkinje cells up to 1 month of age (Kaslin et al., 2013). To determine if juvenile fish can regenerate Purkinje cells after injury, we performed lesion experiments similar to those in the adults (Fig. 4A). To label cells produced after injury, fish were given a 24-h BrdU pulse at day 5, 7 and 10 after the lesion. The fish were then sacrificed at 1.5 and 3 months after the lesion, and examined for colocalisation of BrdU and Purkinje cell markers. Three months after injury we detected BrdU and ZII double-positive Purkinje cells at the lesion site (Fig. 4B,C; n=6, 3.23±2.42 $BrdU^+/ZII^+$ cells).

Lineage tracing of VZ progenitors in the cerebellum during homeostasis and after injury

To identify the contribution of the *ptf1a* lineage after injury and further examine the cellular behaviour of the VZ progenitor population we performed conditional genetic lineage tracing experiments in juvenile and adult zebrafish. We created a driver line with Cherry and the tamoxifen-inducible Cre^{ERT2} recombinase driven by the 5.5 kb 5′ fragment of the *ptf1a* promoter that we previously used to drive *DsRed* expression: *Tg(ptf1a:cherryT2aCreERT2)*[1] (Hans et al., 2009; Kaslin et al., 2013) (Fig. S3A-C). As a reporter, we used a temperature-inducible line that drives EGFP after excision of a loxP-flanked *DsRed* stop sequence: *Tg(hsp70l:DsRed2(floxed)EGFP)* (Hans et al., 2011; Kroehne et al., 2011). Recombination in the *Tg(ptf1a:cherryT2aCreERT2)*[1]×*Tg(hsp70l:DsRed2(floxed)EGFP)* line was induced by incubating embryos in 4-hydroxytamoxifen (4-OH) and adults with tamoxifen. No recombination was detected in the absence of tamoxifen in embryonic or adult zebrafish (Fig. S3D,E). In agreement with previous studies on cerebellar neurogenesis (Volkmann et al., 2008; Bae et al., 2009; Kaslin et al., 2013), recombination between 1 and 3 days postfertilisation resulted in a multitude of GFP-labelled cells in all cell layers of the cerebellum, including PV^+ Purkinje cells, Golgi cells and granule cells (n=12 fish, 10/12 recombined fish; Fig. 4D,E). Furthermore, early recombination resulted in complete unilateral labelling of the neuroepithelial stem cell domain and granule cells (detected in 3/12 recombined fish; Fig. 4E), suggesting that cells produced in one hemisphere do not cross the midline. Later recombination, from day 5 to 6 or at 1 month and analysis 1 or 2 months later, resulted in sparse mosaic labelling of radial glia-like cells, interneurons and Purkinje cells (Fig. 4F,G, Fig. S4A). In fish recombined from day 5 to 6, clones of PV^- interneurons were found around the radial glia-like cells at the VZ (6 clones consisting of 9.2±3.7 cells, n=10; Fig. 4F). By contrast, no clones of cells were detected in proximity to labelled radial glia-like cells in fish that were recombined at 1 month and analysed 3 months later, suggesting that the cells were relatively quiescent (4 clones analysed, n=11; Fig. 4G). Tamoxifen induction resulted in very low recombination rates in adult zebrafish in agreement with the very low number of Ptf1a-expressing cells (<1 cell per section; Fig. 2F). One month after recombination, scarce PV^- cells with stellate and Golgi cell-like morphologies were the only recombined cell type detected in the cerebellum (4.7±2.3 recombined cells/brain, n=24; Fig. S4B). In summary, the lineage tracing experiments demonstrated that the production of cells from the *ptf1a* cell lineage is rare and limited to interneurons beyond 1 month of age.

To identify the contribution of the *ptf1a* lineage after injury in juvenile zebrafish (1 month), recombination was induced by three consecutive tamoxifen incubations 1 day before the lesion. One month after the injury and recombination the fish were heat shocked for 3 days to reveal the recombined cells and their progeny. In agreement with the BrdU pulse-chase experiments, GFP^+/PV^+ Purkinje cells and GFP^+/PV^- Golgi and stellate cells were detected in the cerebellum of tamoxifen-treated fish (Fig. 4H,I; n=14, 0.33 ±0.56 GFP^+/PV^+ cells, 1.64±2.34 GFP^+/PV^- cells). To confirm the results obtained from BrdU pulse-chase experiments in the adult cerebellum (e.g. an inability to regenerate Purkinje cells), we

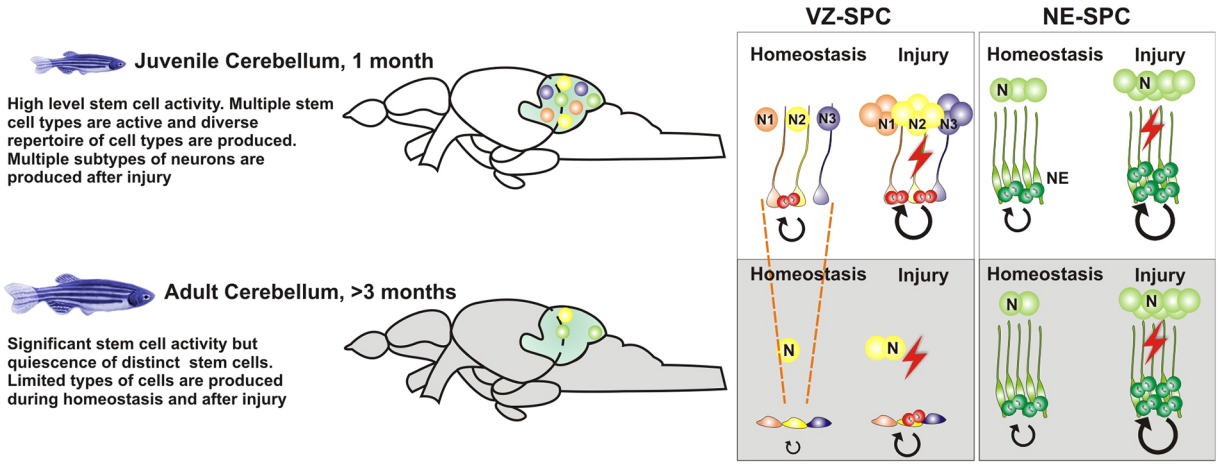

Fig. 5. The diversity of stem cells is lost in the cerebellum during the transition from juvenile to adult and has an impact for homeostatic and regenerative neurogenesis. The juvenile zebrafish maintains radial glia-like VZ and neuroepithelial-like stem and progenitor cells (VZ-SPC and NE-SPC) and is able to produce all major cell types during homeostasis and after injury. By contrast, the adult cerebellum does not produce all cell lineages and is not able to produce all cell types after injury. In the adult cerebellum, the VZ-SPCs become quiescent, whereas NE-SPCs are maintained continuously and contribute to granule cell neurogenesis. N, neurons; NE, neuroepithelial-like cell.

Development (2017) 144, 1462-1471 doi:10.1242/dev.144907

performed recombination experiments in 3-month-old zebrafish after injury. Similar to uninjured adult fish, the recombination rate was low in the injured adult fish (1-3/cells brain, n=30 fish). An absence of PV/ZFII staining indicated that we only detected non-Purkinje GFP$^+$ recombined cells (37 recombined cells analysed, n=30; Fig. S4C), demonstrating that interneurons recover successfully. The number of analysed cells was relatively low but the findings are consistent with the morphological analysis, BrdU pulse-chase experiments, and the abundance and activity of VZ progenitor cells.

DISCUSSION

Implications of heterogeneous stem and progenitor cells in the vertebrate brain

Our findings challenge the assumption that the highly regenerative zebrafish model may be able to regenerate all cell types in the CNS (Fig. 5). It is well established that stem cells are heterogeneous in the vertebrate brain (Shen et al., 2006; Merkle et al., 2007; Kaslin et al., 2009; Ganz et al., 2010; Marz et al., 2010) but the implications of this have yet to be uncovered. The zebrafish telencephalon regenerates well and the cerebellum can regenerate many, but not all, cell types. This implies differences in the regenerative potential of the telencephalon and cerebellum. The circuitry and cell subtypes of the zebrafish telencephalon are not known in detail and so we cannot rule out the possibility that some cell types regenerate poorly in the telencephalon. Another possibility is that, in contrast to the cerebellum, all stem/progenitor cell types are maintained in the adult telencephalon and consequently they are able to produce all the cell lineages required after injury. Future studies revealing new neuronal subpopulations and connections in the telencephalon will support either of these alternatives. A possible difference between the telencephalon and cerebellum is that early-born long projecting neurons (eurydendroid cells) were injured in the cerebellum. By contrast, in the telencephalon it is interneurons that are mostly, if not solely, injured. Interneurons regenerate well in both the cerebellum and telencephalon. It is not clear if projecting neurons regenerate in the telencephalon. Typically, projecting neurons are produced early during development in vertebrates and interneurons later, highlighting the possibility that it is more challenging to regenerate early-produced cell lineages (Carletti and Rossi, 2008; Gage and Temple, 2013). This aligns well with post-embryonic and adult neurogenesis in vertebrates, where only interneurons are produced in the adult.

Regenerative potential in zebrafish reflects adult neurogenesis

In the zebrafish brain, radial glia-like stem and progenitor cells are thought to be the predominant stem cell type in homeostasis and after injury (Kroehne et al., 2011; Kizil et al., 2012; Barbosa et al., 2015; Becker and Becker, 2015; Than-Trong and Bally-Cuif, 2015). Here, we report that radial glia-like cells play only a minor part in adult cerebellar neurogenesis and in recovery after injury. By contrast, we find that the neuroepithelial-like cells are the main stem cell type in the adult cerebellum. The cerebellar stem cells arise early during embryonic development from a common ventricular domain in the hindbrain (Kaslin et al., 2013). The neuroepithelial-like stem cells are derived from the dorsal part of the VZ, and the VZ progenitor cells are derived from the ventral part of the ventricle. The neuroepithelial-like stem cells remain active in the aging cerebellum, where they continuously produce substantial numbers of granule cells throughout life. By contrast, the activity of *ptf1a*-expressing VZ progenitor cells is largely exhausted in the adult cerebellum, and the loss correlates with the very rare genesis of interneurons, Bergmann glia and oligodendrocytes. The post-embryonic VZ progenitor cells in the zebrafish cerebellum share many similarities with the recently identified bi-potent astroglial progenitors in the mouse cerebellar white matter (Parmigiani et al., 2015). Furthermore, other VZ progenitor cell-derived cell types, such as Purkinje and eurydendroid cells, are not produced beyond juvenile stages. The juvenile-to-adult shift in the repertoire of cells produced is accompanied by expression of Pax2 by the VZ progenitor cells at late juvenile stages in zebrafish (Kaslin et al., 2013). Pax2 is required for the differentiation of Golgi and stellate cells in the mouse cerebellum (Maricich and Herrup, 1999). This implies that the potential of the VZ progenitor cells becomes more restricted over time, and is intrinsically controlled by the expression of transcription factors that define the output. Consistent with this notion, the capacity to regenerate granule cells is high in adult zebrafish, whereas the capacity to regenerate interneurons or Purkinje and eurydendriod cells is very low or absent following injury. By contrast, the juvenile cerebellum can regenerate Purkinje cells after injury. A plausible explanation for the ability of juvenile fish to regenerate Purkinje cells is the abundance of VZ progenitors and the capability to produce Purkinje cells during homeostasis (Kaslin et al., 2009, 2013). Given their closely related developmental origin, it is remarkable that the adult cerebellar stem and progenitor cells only appear to be able to contribute to their homeostatic cell lineages after injury (e.g. granule cells or inhibitory interneurons), suggesting that the injury-induced signals are not sufficient to reprogram the stem cells or the environment to enable regeneration of all cerebellar cell types. Taken together, the regenerative potential in the adult cerebellum largely reflects adult cerebellar neurogenesis.

Exhaustion of stem and progenitor cells limits cerebellar regeneration

We previously found that VZ progenitor cells become quiescent, or alternatively exhausted (terminally differentiate or die), in the adult cerebellum (Kaslin et al., 2013). In agreement, recent imaging and lineage tracing studies in the telencephalon of rodents and zebrafish suggest that the neural stem and progenitor cell pool is gradually exhausted over time and that this may impact neural regeneration (Encinas et al., 2011; Rothenaigner et al., 2011; Barbosa et al., 2015; Calzolari et al., 2015). In the adult zebrafish cerebellum, we detected several-fold activation of VZ progenitors after injury, suggesting that quiescent cells are activated. However, the available pool of cells might be limiting because the total number of activated cells was very low (<5 cells/section), indicating that the population is largely exhausted in the adult. In agreement with the limited number of activated VZ progenitors, we only detected modest regeneration of inhibitory interneurons. Taken together, our data suggest that the neural stem cell pool is irreversibly depleted over time in the zebrafish cerebellum, and that this has significant consequences for tissue repair.

Conclusions

The widespread neurogenesis along the brain axis in adult zebrafish could form the basis of powerful new models for studying brain regeneration and neuronal stem cell diversity. The intriguing finding that zebrafish can regenerate some neuronal types, but not others, opens up new possibilities to tease out the molecular mechanisms that allow and restrict brain regeneration and neurogenesis in the vertebrate brain. Moreover, our results illustrate the importance of studying the role of stem cell heterogeneity in the brain. Understanding the molecular basis of *in vivo* neural stem cell

Development (2017) 144, 1462-1471 doi:10.1242/dev.144907

heterogeneity might be of great relevance for future therapeutic approaches.

MATERIALS AND METHODS

Zebrafish

Zebrafish were bred and maintained according to standard procedures (Brand et al., 2002). All animal procedures were approved by the Regierungspräsidium Dresden (permit AZ 24D-9168.11-1/2008-1 and -4) and Monash Animal Ethics Committee (MARP/2013/096) in accordance with the requirements of the National Health & Medical Research Council of Australia. Wild-type experimental fish were from the *gol-b1* line in the AB background. Fish were raised at a density of 50-60 fish/tank. The larvae/juveniles were kept in 7-liter mouse cages and moved to 11-liter glass tanks at 1 month of age. Fish of either sex were used. For the quantifications and lesion experiments, 1-month-old juvenile fish had a body length of 9 ±0.5 mm, 3-month-old young adults were 19±1 mm, and 6-month-old adult fish were 30±2 mm.

Cerebellar injury assay

Cerebellar lesions were made under anaesthesia by drilling a hole in the neurocranium (trepanation) with a vibration-free dental drill (WPI), followed by careful removal of cerebellar tissue. In the 6-month-old adult fish, the lesion results in unilateral removal of a ∼350 µm wide and ∼500 µm deep column of cerebellar tissue. In the 1-month-old juveniles a ∼100 µm wide and ∼200 µm deep column of cerebellar tissue was removed.

Transgenic lines

The *Tg(-5.5ptf1a:DsRed)*, *Tg(nestin:GFP)* and *Tg(hsp70l:DsRed2(floxed)EGFP)* lines have been described previously (Kaslin et al., 2009, 2011, 2013). The *Tg(ptf1a:cherryT2aCreERT2)* line was made by cloning *cherryT2aCreERT2* in place of *DsRed* in the previously used *-5.5ptf1a:DsRed* construct (Kaslin et al., 2013). Stable transgenic lines were created by Tol2-mediated transgenesis and the pT2AL200R150G vector (Kawakami et al., 2004; Urasaki et al., 2006). 25 pg vector DNA and 50 pg Tol2 transposase mRNA were injected into fertilised eggs at the one-cell stage. F0 were raised and incrossed. F1 progeny were identified by screening for Cherry expression.

Tamoxifen induction and lineage tracing

Recombination in zebrafish embryos was achieved by incubating the fish for 24-72 h in embryo medium 3 (E3) containing 5 µM 4-OH and 0.1% DMSO. Recombination in juvenile fish was induced by incubating the fish three times overnight (14 h) in 5 µM tamoxifen in E3 containing 0.1% DMSO. In adult zebrafish the recombination efficacy of immersed fish was compared with intraperitoneal or intraventricular injection of tamoxifen or 4-OH. The immersion protocol resulted in more robust recombination and a higher recombination rate. The lesions were performed 1 day after recombination. Unconditional recombination (leakiness) was tested by heat shocking *Tg(ptf1a:cherryT2aCreERT2)*[1]*; Tg(hsp70l:DsRed2(floxed)EGFP)* fish for 3 days. No unconditional recombination was detected in 1-month-old fish (n=15).

BrdU labelling

To label cycling cells, zebrafish were immersed in 7.5 mM 5-bromo-2-deoxyuridine (BrdU; Sigma) solution (Grandel et al., 2006). The BrdU was dissolved in E3 and the pH adjusted to 7.5.

Tissue preparation

Brains were exposed *in situ* and fixed at 4°C overnight in 2-4% paraformaldehyde/0.1 M phosphate buffer (pH 7.5). They were washed twice with PB and transferred for decalcification and cryoprotection to 20% sucrose/20% EDTA in 0.1 M phosphate buffer (pH 7.5). Brains were frozen in 7.5% gelatine/20% sucrose and cut at 16 µm. Sections were stored at −20°C.

Immunohistochemistry

Immunohistochemistry was carried out as previously described (Kaslin et al., 2004). Briefly, primary and secondary antibodies were incubated in PBS with 0.3% Triton X-100 (PBS TX). Tissue sections were incubated in primary antibodies overnight at 4°C and secondary antibodies for 1 h at room temperature. The slides were then washed in PBS TX and mounted. We used primary antibodies to parvalbumin (mouse, Merck, MAB1572; 1/5000), L-plastin (rabbit, kindly provided by Michael Redd, University of Bristol, UK; 1/5000), NeuroD1 (mouse, Abcam, AB60704; 1/2000), HUC/D (Elavl3) (mouse, Thermo Fisher Scientific, A21271, 1/250), PCNA (mouse, Dako Cyto, M0879, clone PC10, 1/1000), S100β (rabbit, Dako Cyto, Z0311; 1/1000, GABA (rabbit, Sigma, A2052; 1/10,000), Zebrin II/Aldolase C (mouse, kindly provided by Richard Hawkes, University of Calgary, Canada; 1/250), Pax2 (rabbit, Covance/Biolegend, PRB276P; 1/750), GFP (rabbit or chicken, Thermo Fisher Scientific, A11122 and A10262; 1/1000), DsRed (rabbit, Clontech, 632496; 1/1000) and BrdU (rat, Serotec, MCA2060; 1/500). Alexa Fluor 488-, 555- and 633-conjugated secondary antibodies were used (Thermo Fisher Scientific, A11034, A21429, A31576, A11029, A21424, A31574; 1/750).

Image acquisition and processing

Live embryos or zebrafish were anesthetized with 0.1% Tricaine (Sigma), mounted in 1.5% low melting point agarose in E3 and imaged with a Leica TCS SP5 confocal microscope using HCX APO L 20×/0.5 NA, HCX APO L 40×/0.8 NA, HCX APO L 63×/0.9 NA dipping objectives; other images were taken using HC PL APO CS 20×/0.7 NA, HCX PL APO 40×/1.25 NA and HCX PL APO 63×/1.2 NA objectives. To minimise crosstalk between the channels in multicoloured specimens, sequential image acquisition was performed. The images were processed using ImageJ v.1.44 (http://rsb.info.nih.gov/ij/), Volocity (PerkinElmer) and Adobe Photoshop CS2. Figures were assembled using Adobe Photoshop CS2 and Corel Draw X3.

Behavioural analysis

Automated video tracking of swimming performance was performed by using a ZebraTower setup and ZebraLab software (ViewPoint). The swimming arena was 15 cm in diameter and filled with a 10 cm water column. Fish were accommodated for 1 min in the arena prior to start of recording. Swimming behaviour was recorded for 5 min and distance, speed and pattern were analysed using the ZebraLab software.

Quantification and statistical analysis

We assumed normal distribution of variance among animals used in the experiments. To minimise variation, fish raised from the same cohort were used (siblings) with size difference <10% in length. A minimum of five animals were analysed per experimental condition. The exact number of samples analysed (n) is stated for each experiment in the results section. We quantified the number of labelled cells in every fourth section at 16 µm throughout the entire anterior-posterior length of the cerebellar corpus (8-9 sections analysed in adult fish). The cell size is small in adult zebrafish (typically 6-12 µm) and the section interval (48 µm) makes counting of the same cell in adjacent sections in a series unlikely, and therefore no post-correction method was used to correct for over-representation of cell number. For colocalisation studies with cellular markers, colocalisation was verified by analysing high-resolution confocal stacks. The optical sections were taken with 0.5-1 µm intervals using 40× (1.25 NA) or 63× (1.2 NA) objectives. Using power calculations for 80% power at 5% significance level and s.d. values measured from experiments show requirement of 4-6 animals/group. Comparison between the groups was made by unpaired two-tailed Student's t-test or one-way ANOVA analysis with Tukey's multiple comparisons post-test. Data are presented as mean±s.d. Statistical significance was established at P<0.05. α=0.5, using Prism (v.4.03 and 6, GraphPad).

Acknowledgements

We thank M. Geffarth, A. Machate, S. von Kannen and the Biotec-TUD Zebrafish Facility for excellent technical support; Monash Micro Imaging for technical and imaging support; Monash FishCore for excellent support; and D. Freudenreich, M. Änkö and C. Kizil for comments on the manuscript.

Competing interests

The authors declare no competing or financial interests.

Author contributions

J.K. and M.B. conceptualised, designed and provided resources for the work. J.K. performed experiments, analysed data and wrote the manuscript. J.G., V.K. and S.H. co-performed experiments and commented on the manuscript.

Funding

This work was supported by grants from the Deutsche Forschungsgemeinschaft (SFB 655-A3), European Union (European Research Council AdG Zf-BrainReg) and the German Excellence Initiative of the Bundesministerium für Bildung und Forschung (Institutional Strategy, TU Dresden) to M.B., and Erdi Foundation and National Health and Medical Research Council project grant (GNT1060538), Faculty of Medicine, Nursing and Health Sciences, Monash University strategic grant to J.K. The Australian Regenerative Medicine Institute is supported by funds from the state government of Victoria and the Australian federal government.

Supplementary information

Supplementary information available online at
http://dev.biologists.org/lookup/doi/10.1242/dev.144907.supplemental

References

Adolf, B., Chapouton, P., Lam, C. S., Topp, S., Tannhäuser, B., Strähle, U., Götz, M. and Bally-Cuif, L. (2006). Conserved and acquired features of adult neurogenesis in the zebrafish telencephalon. *Dev. Biol.* **295**, 278-293.

Bae, Y.-K., Kani, S., Shimizu, T., Tanabe, K., Nojima, H., Kimura, Y., Higashijima, S. and Hibi, M. (2009). Anatomy of zebrafish cerebellum and screen for mutations affecting its development. *Dev. Biol.* **330**, 406-426.

Barbosa, J. S., Sanchez-Gonzalez, R., Di Giaimo, R., Baumgart, E. V., Theis, F. J., Götz, M. and Ninkovic, J. (2015). Neurodevelopment. Live imaging of adult neural stem cell behavior in the intact and injured zebrafish brain. *Science* **348**, 789-793.

Baumgart, E. V., Barbosa, J. S., Bally-Cuif, L., Götz, M. and Ninkovic, J. (2012). Stab wound injury of the zebrafish telencephalon: a model for comparative analysis of reactive gliosis. *Glia* **60**, 343-357.

Becker, C. G. and Becker, T. (2015). Neuronal regeneration from ependymo-radial glial cells: cook, little pot, cook!. *Dev. Cell* **32**, 516-527.

Becker, T., Wullimann, M. F., Becker, C. G., Bernhardt, R. R. and Schachner, M. (1997). Axonal regrowth after spinal cord transection in adult zebrafish. *J. Comp. Neurol.* **377**, 577-595.

Brand, M., Granato, M. and Nüsslein-Volhard, C. (2002). Keeping and raising zebrafish. In *Zebrafish: A Practical Approach* (ed. C. Nüsslein-Volhard and R. Dahm). Oxford: Oxford University Press.

Calzolari, F., Michel, J., Baumgart, E. V., Theis, F., Götz, M. and Ninkovic, J. (2015). Fast clonal expansion and limited neural stem cell self-renewal in the adult subependymal zone. *Nat. Neurosci.* **18**, 490-492.

Carletti, B. and Rossi, F. (2008). Neurogenesis in the cerebellum. *Neuroscientist* **14**, 91-100.

Encinas, J. M., Michurina, T. V., Peunova, N., Park, J.-H., Tordo, J., Peterson, D. A., Fishell, G., Koulakov, A. and Enikolopov, G. (2011). Division-coupled astrocytic differentiation and age-related depletion of neural stem cells in the adult hippocampus. *Cell Stem Cell* **8**, 566-579.

Fitch, M. T. and Silver, J. (2008). CNS injury, glial scars, and inflammation: inhibitory extracellular matrices and regeneration failure. *Exp. Neurol.* **209**, 294-301.

Gage, F. H. and Temple, S. (2013). Neural stem cells: generating and regenerating the brain. *Neuron* **80**, 588-601.

Ganz, J., Kaslin, J., Hochmann, S., Freudenreich, D. and Brand, M. (2010). Heterogeneity and Fgf dependence of adult neural progenitors in the zebrafish telencephalon. *Glia* **58**, 1345-1363.

Grandel, H., Kaslin, J., Ganz, J., Wenzel, I. and Brand, M. (2006). Neural stem cells and neurogenesis in the adult zebrafish brain: origin, proliferation dynamics, migration and cell fate. *Dev. Biol.* **295**, 263-277 *These authors contributed equally.

Hans, S., Kaslin, J., Freudenreich, D. and Brand, M. (2009). Temporally-controlled site-specific recombination in zebrafish. *PLoS ONE* **4**, e4640.

Hans, S., Freudenreich, D., Geffarth, M., Kaslin, J., Machate, A. and Brand, M. (2011). Generation of a non-leaky heat shock-inducible Cre line for conditional Cre/lox strategies in zebrafish. *Dev. Dyn.* **240**, 108-115.

Hoshino, M., Nakamura, S., Mori, K., Kawauchi, T., Terao, M., Nishimura, Y. V., Fukuda, A., Fuse, T., Matsuo, N., Sone, M. et al. (2005). Ptf1a, a bHLH transcriptional gene, defines GABAergic neuronal fates in cerebellum. *Neuron* **47**, 201-213.

Kani, S., Bae, Y.-K., Shimizu, T., Tanabe, K., Satou, C., Parsons, M. J., Scott, E., Higashijima, S. and Hibi, M. (2010). Proneural gene-linked neurogenesis in zebrafish cerebellum. *Dev. Biol.* **343**, 1-17.

Kaslin, J. and Brand, M. (2012). Cerebellar development and neurogenesis in zebrafish. In *Handbook of the Cerebellum and Cerebellar Disorders* (ed. M. Manto, D. L. Gruol, J. D. Schmahmann, N. Koibuchi and F. Rossi). Berlin: Springer.

Kaslin, J., Nystedt, J. M., Ostergard, M., Peitsaro, N. and Panula, P. (2004). The orexin/hypocretin system in zebrafish is connected to the aminergic and cholinergic systems. *J. Neurosci.* **24**, 2678-2689.

Kaslin, J., Ganz, J. and Brand, M. (2008). Proliferation, neurogenesis and regeneration in the non-mammalian vertebrate brain. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **363**, 101-122.

Kaslin, J., Ganz, J., Geffarth, M., Grandel, H., Hans, S. and Brand, M. (2009). Stem cells in the adult zebrafish cerebellum: initiation and maintenance of a novel stem cell niche. *J. Neurosci.* **29**, 6142-6153.

Kaslin, J., Kroehne, V., Benato, F., Argenton, F. and Brand, M. (2013). Development and specification of cerebellar stem and progenitor cells in zebrafish: from embryo to adult. *Neural Dev.* **8**, 9.

Kawakami, K., Takeda, H., Kawakami, N., Kobayashi, M., Matsuda, N. and Mishina, M. (2004). A transposon-mediated gene trap approach identifies developmentally regulated genes in zebrafish. *Dev. Cell* **7**, 133-144.

Kernie, S. G. and Parent, J. M. (2010). Forebrain neurogenesis after focal ischemic and traumatic brain injury. *Neurobiol. Dis.* **37**, 267-274.

Kishimoto, N., Shimizu, K. and Sawamoto, K. (2012). Neuronal regeneration in a zebrafish model of adult brain injury. *Dis. Model. Mech.* **5**, 200-209.

Kizil, C., Kaslin, J., Kroehne, V. and Brand, M. (2012). Adult neurogenesis and brain regeneration in zebrafish. *Dev. Neurobiol.* **72**, 429-461.

Kroehne, V., Freudenreich, D., Hans, S., Kaslin, J. and Brand, M. (2011). Regeneration of the adult zebrafish brain from neurogenic radial glia-type progenitors. *Development* **138**, 4831-4841.

Kyritsis, N., Kizil, C., Zocher, S., Kroehne, V., Kaslin, J., Freudenreich, D., Iltzsche, A. and Brand, M. (2012). Acute inflammation initiates the regenerative response in the adult zebrafish brain. *Science* **338**, 1353-1356.

Liu, F., You, Y., Li, X., Ma, T., Nie, Y., Wei, B., Li, T., Lin, H. and Yang, Z. (2009). Brain injury does not alter the intrinsic differentiation potential of adult neuroblasts. *J. Neurosci.* **29**, 5075-5087.

Magnusson, J. P., Goritz, C., Tatarishvili, J., Dias, D. O., Smith, E. M. K., Lindvall, O., Kokaia, Z. and Frisen, J. (2014). A latent neurogenic program in astrocytes regulated by Notch signaling in the mouse. *Science* **346**, 237-241.

Maricich, S. M. and Herrup, K. (1999). Pax-2 expression defines a subset of GABAergic interneurons and their precursors in the developing murine cerebellum. *J. Neurobiol.* **41**, 281-294.

Marz, M., Chapouton, P., Diotel, N., Vaillant, C., Hesl, B., Takamiya, M., Lam, C. S., Kah, O., Bally-Cuif, L. and Strahle, U. (2010). Heterogeneity in progenitor cell subtypes in the ventricular zone of the zebrafish adult telencephalon. *Glia* **58**, 870-888.

Merkle, F. T., Mirzadeh, Z. and Alvarez-Buylla, A. (2007). Mosaic organization of neural stem cells in the adult brain. *Science* **317**, 381-384.

Parmigiani, E., Leto, K., Rolando, C., Figueres-Onate, M., Lopez-Mascaraque, L., Buffo, A. and Rossi, F. (2015). Heterogeneity and bipotency of astroglial-like cerebellar progenitors along the interneuron and glial lineages. *J. Neurosci.* **35**, 7388-7402.

Reimer, M. M., Sorensen, I., Kuscha, V., Frank, R. E., Liu, C., Becker, C. G. and Becker, T. (2008). Motor neuron regeneration in adult zebrafish. *J. Neurosci.* **28**, 8510-8516.

Rothenaigner, I., Krecsmarik, M., Hayes, J. A., Bahn, B., Lepier, A., Fortin, G., Gotz, M., Jagasia, R. and Bally-Cuif, L. (2011). Clonal analysis by distinct viral vectors identifies bona fide neural stem cells in the adult zebrafish telencephalon and characterizes their division properties and fate. *Development* **138**, 1459-1469.

Shen, Q., Wang, Y., Dimos, J. T., Fasano, C. A., Phoenix, T. N., Lemischka, I. R., Ivanova, N. B., Stifani, S., Morrisey, E. E. and Temple, S. (2006). The timing of cortical neurogenesis is encoded within lineages of individual progenitor cells. *Nat. Neurosci.* **9**, 743-751.

Than-Trong, E. and Bally-Cuif, L. (2015). Radial glia and neural progenitors in the adult zebrafish central nervous system. *Glia* **63**, 1406-1428.

Urasaki, A., Morvan, G. and Kawakami, K. (2006). Functional dissection of the Tol2 transposable element identified the minimal cis-sequence and a highly repetitive sequence in the subterminal region essential for transposition. *Genetics* **174**, 639-649.

Volkmann, K., Rieger, S., Babaryka, A. and Köster, R. W. (2008). The zebrafish cerebellar rhombic lip is spatially patterned in producing granule cell populations of different functional compartments. *Dev. Biol.* **313**, 167-180.