RESEARCH ARTICLE

STEM CELLS AND REGENERATION

Exclusive multipotency and preferential asymmetric divisions in post-embryonic neural stem cells of the fish retina

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ABSTRACT

The potency of post-embryonic stem cells can only be addressed in the living organism, by labeling single cells after embryonic development and following their descendants. Recently, transplantation experiments involving permanently labeled cells revealed multipotent neural stem cells (NSCs) of embryonic origin in the medaka retina. To analyze whether NSC potency is affected by developmental progression, as reported for the mammalian brain, we developed an inducible toolkit for clonal labeling and non-invasive fate tracking. We used this toolkit to address post-embryonic stem cells in different tissues and to functionally differentiate transient progenitor cells from permanent, bona fide stem cells in the retina. Using temporally controlled clonal induction, we showed that post-embryonic retinal NSCs are exclusively multipotent and give rise to the complete spectrum of cell types in the neural retina. Intriguingly, and in contrast to any other vertebrate stem cell system described so far, long-term analysis of clones indicates a preferential mode of asymmetric cell division. Moreover, following the behavior of clones before and after external stimuli, such as injuries, shows that NSCs in the retina maintained the preference for asymmetric cell division during regenerative responses. We present a comprehensive analysis of individual post-embryonic NSCs in their physiological environment and establish the telost retina as an ideal model for studying adult stem cell biology at single cell resolution.

KEY WORDS: Neural stem cells, Neural progenitor cells, Multipotency, Asymmetric division, Retina, Medaka

INTRODUCTION

Embryonic progenitor and stem cells generate new differentiated cells during the initial phases of development. Post-embryonic stem cells deal with a rather different issue, which is the addition of new cells to already functional organs. The switch from embryonic to post-embryonic stem cells is in general accomplished by loss of potency: while pluripotent cells can generate all cell types during early embryogenesis, adult organs and tissues are usually maintained by lineage-restricted post-embryonic stem cells (Blanpain and Fuchs, 2009; Van Keymeulen et al., 2011; Zhu et al., 2011).

Fish display a unique feature among vertebrates, which is their constant allometric growth beyond sexual maturity. All cell types are constantly added to every organ, making fish an ideal model to study post-embryonic stem cells and particularly how stem cell potency is affected during the transition from early embryo to juvenile and adult. Stem cell lineage analysis in fish has been largely performed by transplantation or by DNA injection, but both methods require the analysis of hundreds of clones to exclude putative technical artifacts (Centanin et al., 2011; Tu and Johnson, 2011; Wong and Rapaport, 2009). By contrast, genetic labeling of individual cells among a population (Livet et al., 2007) has proven to be an extremely useful, non-invasive tool for the analysis of embryonic and post-embryonic stem cells (Bonaguidi et al., 2011; Gupta and Poss, 2012; Rinkevich et al., 2011; Snippert et al., 2010; Loulier et al., 2014). Cre-mediated recombination was recently validated in fish (Gupta and Poss, 2012; Hans et al., 2009; Knopf et al., 2011; Mosimann et al., 2011; Nakamura et al., 2010; Singh et al., 2012), allowing long-term lineage of stem cells of embryonic origin (Pan et al., 2013).

The fish neural retina (NR) is ideally suited for comprehensively studying individual NSCs in a post-embryonic organ due to its stereotypic cell type distribution and spatio/temporal organization. It consists of seven main cell types distributed in three nuclear layers, and all these cell types are added continuously from the peripheral ciliary marginal zone (CMZ) (Amato et al., 2004; Johns, 1977; Reh and Levine, 1998; Centanin and Wittbrodt, 2014), which constitutes the niche of retinal stem cells (RSCs). In medaka, retinal NSCs were identified by the formation of arched continuous stripes (ArCoSs) (Centanin et al., 2011). In these experiments, permanently labeled blastula cells were transplanted into unlabeled blastula hosts. The formation of labeled ArCoSs containing all cell types of the neural retina demonstrated the existence of multipotent retinal NSCs of embryonic origin. However, early transplantation experiments do not allow the study of how changes in potency may occur after embryonic development due to the potential changes in niche and organ function. Particularly, the issue of whether cells in the post-embryonic neural retina are produced by multipotent NSCs or by the combined activity of several lineage-restricted – although still clonally related – NSCs could not be assessed with the described approach.

Here, we present a ‘living’ toolkit for individual post-embryonic stem cell research in medaka, and use it to address the localization and potency of post-embryonic NSCs with single cell resolution in vivo in their organismal context. Using inducible drivers for Cre recombinase, we demonstrate that post-embryonic NSCs always generate all cell types of the neural retina, including neurons and glia. Additionally, by labeling individual post-embryonic NSCs in...
the retina and following the resulting clone, we demonstrate a preferential asymmetric mode of cell division that is not changed after external challenges.

RESULTS

A medaka toolkit for life-long lineage analysis of individual stem cells

To address individual post-embryonic stem cells, we developed a toolkit based on Brainbow constructs (Livet et al., 2007; Pan et al., 2013) that allows the induction of colorful mosaic medaka fish suitable for long-term lineage analysis (Fig. 1A,B). This living toolkit was named Gaudí after the Spanish architect famous for his colorful mosaics (supplementary material Fig. S1), and is composed of two alternative transgenic lines for inducible Cre expression and three fluorescent reporter lines to follow lineages (see Materials and Methods).

Gaudí\textsuperscript{HspCRE.A} (Fig. 1A, top) contains a nuclear-tagged Cre recombinase, the expression of which is inducible upon heat-shock treatment until 10 days post-fertilization (\textit{Hsp70\textsubscript{-nlsCRE}}). Gaudí\textsuperscript{Ubiquin\textsubscript{ER2}Cre} (Fig. 1A, bottom) contains a tamoxifen-inducible Cre recombinase under the control of a ubiquitous promoter (\textit{ubiquitin\textsubscript{-ERT2}Cre}). For both lines, recombinase activity in most tissues is only detectable after induction (Figs 2 and 3; supplementary material Fig. S2).

Gaudí\textsuperscript{RSG} (Gaudí Red-Switch-Green; Fig. 1B, top) ubiquitously expresses a floxed DS-Red fluorescent protein, which prevents the expression of a nuclear-tagged EGFp. After Cre induction, the H\textsubscript{2}B-EGFP is evident in all recombined cells (Fig. 2A) and inherited by their progeny.

Gaudí\textsuperscript{BBW2.1} (Gaudí Brainbow 2.1; Fig. 1B, middle) offers additional fluorescent read-out for recombination. Upon Cre expression, the default membrane-tagged ECFP switches to one out of three alternative FPs: YFP, dTomato or \textsuperscript{a}EGFP (see supplementary material Fig. S2). This greatly expands the number of individual cells that can be followed within a tissue of interest.

Gaudí\textsuperscript{LBBB} (Gaudí floxed DSRd, Brainbow 2.1; Fig. 1B, bottom) ubiquitously expresses a DS-Red FP, which is floxed-out upon Cre activation, allowing the expression of one out of four FPs: \textsuperscript{a}EGFP, YFP\textsubscript{mem}ECFP and dTomato (Fig. 2B-D). Gaudí\textsuperscript{LBBB} is the best option when fixation and immunostaining are required, as a single α-GFP antibody can be used to recognize three FP outputs based on their differential subcellular localization (Fig. 2C,D).

The Gaudí toolkit permits labeling cells and lineage analysis of stem cells in most medaka tissues

To perform a proper lineage analysis, the reporter lines for recombination (\textit{LoxP}-containing Gaudí lines, in this case) have to be expressed in every tissue and in every cell type of the organism, and the expression has to be maintained during the total chase or lineage time. Otherwise, the lineage will constitute only a fraction of the entire progeny, and the real potency of the stem cells studied will be underestimated. We detected the expression of the default or the alternative recombination read-out (fluorescent proteins expressed after Cre activation) in every embryonic and post-embryonic organ of the Gaudí reporter lines (Figs 1B, 2 and 3; supplementary material Fig. S3).

Both Gaudí\textsuperscript{HspCRE.A} and Gaudí\textsuperscript{Ubiquin\textsubscript{ER2}Cre} drive recombination in the CMZ (Fig. 3A), and in many other tissues such as the cornea, brain, somites, intestine, lateral line, epidermis and gills (Fig. 3B-H). One of the benefits of these inducible driver lines is that recombination levels can be adjusted by regulating the dose of the inducer (shift in temperature for Gaudí\textsuperscript{HspCRE.A} and tamoxifen exposure for Gaudí\textsuperscript{Ubiquin\textsubscript{ER2}Cre} (Bonaguidi et al., 2011). The experimental advantage of fish is their external development and the transparency of specific tissues even during larval stages, permitting screening under a fluorescence stereomicroscope. Using the Gaudí toolkit, the levels of recombination observed in the somites \textit{in vivo} 2 days after induction (Fig. 3I-K) are a good proxy of the recombination that took place in the retina (Fig. 3L-N).

We used this selection criterion for the experiments performed here, and rely on either sparse recombination (Bonaguidi et al., 2011) [in the case of the Gaudí\textsuperscript{RSG} line (see Material and Methods section)] or on just one of the possible read-outs in Gaudí\textsuperscript{BBW2.1} to reach clonality.

To validate the Gaudí toolkit as an appropriate method for lineage analysis \textit{in vivo}, we compared it with alternative and already validated ways to label lineages in fish, namely DNA injection into the 2- to 4-cell stage embryos (Tu and Johnson, 2011) and transplantation at blastula stages (Centanin et al., 2011). Clones generated after DNA injection or transplantation experiments
(supplementary material Fig. S4) reveal the lineage of cells labeled during early embryonic development. Induction of recombination at early embryonic stages using either GaudíHspCre.A or GaudíUbiq.iCre in combination with Gaudí reporter lines resulted in the very same output (supplementary material Fig. S4A-C), as expected for a toolkit driving efficient labeling and allowing long-term lineage in vivo.

Definitive statements about stem cells involved in homeostasis (or homeostatic growth in the case of fish) need a late induction of clones, preferentially after functional development of the target organ is completed. We induced clones in Gaudí juvenile fish and grew them to adulthood (supplementary material Fig. S4). The resulting clones resembled the reported outputs for DNA injection and transplantation experiments in well-characterized tissues such as the fin (supplementary material Fig. S4D-G) (Tu and Johnson, 2011; Wong and Rapaport, 2009). The Gaudi kit confirmed the existence of post-embryonic, bona fide stem cells in a variety of organs throughout the entire fish (previous sections, and data not shown) and therefore constitutes a fundamental and versatile tool for the study of fish stem cells.

The Gaudi toolkit reveals post-embryonic NSCs in the fish retina

Neurons are added to the neural retina (NR) of fish in an extremely stereotyped manner and stay in that same place for the rest of the fish’s life, with no mixing among neurons born at different time points (Johns, 1977). This precise spatio/temporal arrangement is referred to as a tree-like growth in concentric rings (Johns, 1977), where the peripheral addition of new neurons results in a central circle that contains the oldest, embryonic retinal cells and peripheral rings that are composed of newly differentiated cells (Fig. 4A). This form of growth was shown in several different fish species, such as goldfish, zebrafish and medaka (Johns, 1977; Allison et al., 2010; Centanin et al., 2011). In medaka, retinal stem cells (RSCs) of embryonic origin form ArCoSs, which are clonal stripes that extend perpendicular to the temporal rings, from the embryonic to the adult
retina (Fig. 4B) (Centanin et al., 2011). Therefore, ArCoS formation is the way of defining stemness for a retinal cell in fish. In medaka, ArCoSs are composed of all seven main neuro-retinal cell types (Centanin et al., 2011).

We decided to use the Gaudí kit to characterize RSCs functionally in a mature retina. After inducing stochastic recombination of Gaudí reporter lines in juvenile fish, those showing recombination in the retina were selected and grown for 3 months. These retinae showed induced ArCoSs (iArCoSs) (n=15/23 retinae, 65%) spanning from the juvenile to the adult retina (n=86 iArCoSs distributed in 15 retinae, averaging 5.7 iArCoSs per retina) (Fig. 4C), highlighting post-embryonic RSCs. In contrast to embryonic ArCoSs, which extend out of the embryonic retina, iArCoSs start at more peripheral positions, revealing their post-embryonic origin (Fig. 4C-E). This confirms that the NR contains genuine post-embryonic stem cells that sustain its growth during the entire life of the fish.

Post-embryonic retinal NSCs and progenitor cells are located in distinct domains of the CMZ
In addition to the permanent RSCs, several studies of the adult teleost CMZ postulate the existence of transient retinal progenitor cells (RPCs) (Johns, 1977; Reh and Levine, 1998), as described in other stem cell niches (Mizutani et al., 2007; Rothenaigner et al., 2011). Notably, it has been especially challenging to functionally differentiate progenitor cells from genuine stem cells in vivo. We took advantage of the temporal arrangement of the retina to examine the site corresponding to the induction time (juvenile retina) (Fig. 4C-F), which can be easily demarcated by performing a short pulse of IdU when Cre-recombinase is induced (Fig. 4D, induction circle). Analysis of adult retinae from Gaudí\textsuperscript{HspCre.A} Gaudí\textsuperscript{BBW2.1} induced at juvenile stages revealed two different features of proliferating retinal cells. Besides iArCoSs spanning from the induction circle to the peripheral retina (filled arrowheads, Fig. 4C), we identified short clones terminating soon after their birth time (45 short clones, n=10 retinae) (open arrowheads, Fig. 4C). These represent clonal footprints of progenitor cells that, due to their transient nature, only give rise to limited progeny. Strikingly, there is a stereotypic spatio/temporal gap between the footprint of progenitor cells and the first cells in an iArCoS (n=17 RPCs footprints and 15 ArCoSs in five retinae). Given the tight spatiotemporal correlation of cell addition during fish retinal growth, this gap indicates that the ‘more central’ footprints of RPCs were incorporated earlier and the ‘more peripheral’ ArCoSs from RSCs started later (Fig. 4C,F; supplementary material Fig. S5, Movies 1 and 2). This in turn reflects the initial positions of RPCs and RSCs in the CMZ at the time of induction. Although RPCs are located more centrally and therefore their progeny exit the CMZ earlier, RSCs reside in an adjacent but more peripheral circle and it takes longer for their progeny to pass through the RPC domain before they are eventually integrated into the differentiated retina (supplementary material Fig. S5).

RSCs and RPCs have different temporal requirements
As the spatio/temporal organization of the retina allows us to address RPCs separately from RSCs, we investigated for how long a RPC produces progeny before it is exhausted. We induced a few individual clones per retina and found that at 7 days post-induction (Fig. 5A,B) the progeny of RPCs was already detached from the CMZ (Fig. 5C,D; supplementary material Movie 1), indicating the exhaustion of
proliferative cells in the clone. Complementarily, the progeny of labeled RSCs took 7 days to fill up the CMZ and to start contributing differentiated cells to the layered retina (Fig. 5E,F; supplementary material Movie 2). Therefore, lineage analysis of single cells in the context of the stereotypic growth occurring in the fish retina makes it possible to define the initiation and exhaustion of the proliferative capacities of RPCs and RSCs in their natural niche.

Both RSCs and RPCs are multipotent, and each individual RSC generates all cell types in the NR

To address the potency and proliferative capacities of RSCs and RPCs, we analyzed the cell number and cellular composition of 

\( i \)ArCoSs and footprints (containing more than 20 cells), respectively. We induced sparse recombination in Gaudí and performed quantifications in the entire retina at 7 dpi. RSCs preferentially generated clones containing more than 200 cells (73.4%, \( n=214 \)) (Fig. 5G), and in all cases these cells filled a complete retinal column distributed in all three nuclear layers. Conversely, most clones generated by RPCs (the footprints mentioned above) ranged from 25 to 99 cells (81.3%, \( n=64 \)) (Fig. 5G), and some expanded to 170 differentiated retinal cells (6.3%, \( n=64 \)). All RPC clones produced more than two cell types, ruling out the existence of dedicated, cell type-specific RPCs in the mature retina. This extends reports on RPC potential performed during fish retinogenesis (He et al., 2012) and the early frog CMZ (Wetts et al., 1989; Wong and Rapaport, 2009).

To address the full potency of retinal stem cells in the mature CMZ, we analyzed the cellular composition of 

\( i \)ArCoSs 2 months post-induction. The Gaudi toolkit in combination with the stereotyped organization of cell types in the NR allows us to study the potency of hundreds of individual RSCs (Fig. 6A-C). Lineage analyses indicated that every single NSC analyzed in the retina is multipotent (>300) with progeny distributed over the three nuclear layers (Fig. 6D), as previously seen for shorter lineage times (Fig. 5E,F; supplementary material Movie 3). Even neighbor clones maintained by adjacent RSCs labelled by different outputs of the
Gaudí LRW were composed of cells that fill the entire retinal layers (Fig. 6E,E′). To confirm that RSCs exclusively generate iArCoSs that contain every main cell type of the neural retina, we used specific antibodies against cell types in the inner nuclear layer. We found that all iArCoSs analyzed were positive for the antibodies tested (n=97 iArCoSs; 63/63 PKCa+, bipolar cells; 77/77 GS+, Müller glia cells; 18/18 HuC+, horizontal and amacrine cells; 20/20 parvalbumin+, amacrine cells; 9/9 triple positives for GS, PKCa and HuC; data not shown). This demonstrates that each iArCoS-forming RSC self-renews and always gives rise to the entire complement of neural retinal main cell types.

**Preferential asymmetric cell divisions of NSCs in the medaka retina**

Recently, a number of reports analyzing diverse vertebrate post-embryonic stem cells at clonal resolution (Doupé et al., 2012; Klein et al., 2010; Mascré et al., 2012; Snippert et al., 2010) shifted the traditional view on stem cell behavior in vivo (Simons and Clevers, 2011). These studies sustain a model of homeostatic turnover in which equally potent stem cells undergo a continuous process of stochastic loss and replacement so that their overall population is maintained. Through this process of neutral competition, stem cells marked in a pulse-labeling assay become increasingly invested in an ever-diminishing population of surviving clones (see the expected progression of RSCs clones according to this model in Fig. 7A,B). Intriguingly, retinal NSCs in fish give rise to iArCoSs that are not lost nor do they displace other clones over time (Fig. 7C-G; see also Fig. 6C). Instead, iArCoSs are stable throughout the life of the animal (98% of iArCoSs generated in juvenile fish continue into the adult CMZ, n>200). These results suggest that retinal NSCs are maintained through a predominantly asymmetric mode of cell division.

This preferential mode of asymmetric divisions is maintained by RSCs of the NR throughout life. If there was a shift to symmetric divisions at some point during late adulthood, we would expect to observe a more stochastic scenario (Fig. 7A,B), i.e. the termination of some iArCoSs and the widening of others. Even in the oldest fish analyzed (18 months after transplantation or induced recombination), ArCoSs and iArCoSs maintained their continuous, consistently wide shape throughout life (n>60 ArCoSs, n>50 iArCoSs).

**RSCs keep their division mode after injuries in the transient amplifying domain**

Stem cell decisions taking place during homeostatic conditions can be altered when challenged by injuries, a feature widely reported for...
other post-embryonic stem cells (Blanpain and Fuchs, 2009; Van Keymeulen et al., 2011). To investigate whether the preference for asymmetric divisions is maintained in RSCs during regeneration, we used the highly ordered fashion in which cells are added to the fish retina to compare the width of a growing iArCoS before and after injury. Gaudí fish were induced by Cre-mediated recombination and grown until adulthood. (C-E) Individual NSCs in the retina are multipotent. Every clone spans through the three retinal nuclear layers (C) and contains all major retinal cell types (D). Gaudí allows unambiguous assignment of iArCoSs and demonstrates multipotency in adjacent RSCs (E, and detail in E).

Transplantation experiments at blastula stages revealed the presence of about 35 active RSCs in the neural retina at its very initial stage. We analyzed the relative occupancy of 106 ArCoSs in 2-month-old adult fish, and estimated 34.8±2.1 active RSCs present at the earliest retinogetic stage (Fig. 8B,E). The analysis of Gaudí fish induced at a pre-neurogenic retinal stage revealed the presence of 60±4.5 active RSCs. Induction of recombination in Gaudí fish induced at 5 dpf, in which most of the retina is already layered and the CMZ is evident, revealed that the number of active RSCs had increased to 113.1±7.4 (Fig. 8B-E). Moreover, when Gaudí fish were induced at 10 dpf, the number of active RSCs had increased to 113.1±7.4 (Fig. 8B-E). Our data demonstrate that RSCs do indeed go through symmetric divisions to expand the number of active stem cells during eye growth.

**DISCUSSION**

In this study, we developed a genetic toolkit that allows single cell labeling and long-term lineage analysis in medaka to gain insight into the functional properties of retinal stem cells (RSCs) in vivo. We generated and used the Gaudí toolkit to reveal the occurrence of RSCs in an already functional eye, and to demonstrate that individual RSCs generate the entire repertoire of cell types in the neural retina. We show that RSCs preferentially (but not exclusively) divide in an asymmetric way that is reflected in the lifelong clone continuity. RSCs also divide (to a minor extent) in a symmetric way, which increases the stem cell number and sustains the equatorial expansion of the growing retina. Our results indicate two stereotypic behaviors among RSCs in medaka: (1) there is no variation in potency among individual stem cells in the fish neural retina; and (2) the preference for asymmetric divisions is not altered by age or during tissue repair.
Homogenous potential of RSCs in the neural retina

When assessed by single cell labeling methods, progenitor cells have proved to be extremely heterogeneous regarding the number and cell types produced – irrespective of the system in which these were analyzed (Brock et al., 2009; Costa et al., 2011; Graf and Stadtfeld, 2008; Mascre et al., 2012; Rothenaigner et al., 2011). Several studies assessed the potency of individual RPCs in the early retina of different vertebrates (Fekete et al., 1994; Gomes et al., 2011; He et al., 2012; Holt et al., 1988; Price et al., 1987; Wong and Rapaport, 2009; Wettas and Fraser, 1988), and they all reported a scenario in which the number and cell types generated are highly variable among RPCs.

The situation we report for the homeostatic growth of the neural retina is far more uniform. We focused on RPCs with the potential of going through at least six more divisions (i.e. generating clones that contain more than 25 cells) and observed that all of them were multipotent. Furthermore, most of the RPCs analyzed were able to generate all seven main cell types present in the neural retina.

Although our results neither overrule stochasticity during differentiation nor exclude the existence of dedicated RPCs, they clearly indicate that such a behavior would be restricted to the last cell divisions before cells acquire their post-mitotic identity. Our data indicate that a few divisions higher in the lineage, cells of different retinal cell types share a common progenitor cell.

Our short-lineage experiments revealed transient clones containing up to 170 cells. These clones could originate from either RPCs or RSCs that are displaced from the CMZ niche, as shown for stem cells in other systems (Snippert et al., 2010). The fact that these big clones are consistently terminated within a few days indicates a limitation in their proliferative potential. They could still be seen as originating from identical cells and resulting in a different output due to cell competition. However, we reproducibly observed that the terminated clones were closer to the center of the retina than the central edges of ArCoSs, arguing that these terminated clones arise from cells that were originally sited at a more central location in the expanding retina (supplementary material Fig. S5). Therefore, we argue that cells with limited proliferative potential map to a defined more-central domain within the CMZ, the transit amplifying zone, and therefore represent RPCs.

Our data show that the proliferative capacities of stem-cell-derived RPCs cover a wide range, and go beyond the mitotic capacities reported for early RPC during initial retinal neurogenesis in...
zebrafish and Xenopus (He et al., 2012; Holt et al., 1988; Wong and Rapaport, 2009; Wetts and Fraser, 1988).

This multipotent behavior of RPCs is highly reminiscent of that of stem cells in the medaka neural retina. In contrast to individual NSCs in rodents, which generate variable multi-lineage offspring (Bonaguidi et al., 2011; Encinas et al., 2011), we observe fully penetrant multipotency at the level of individual NSCs. This is massively expanding the present view on the potential of a single post-embryonic neural stem cell. Each iArCoS-forming RSC self-renews and always gives rise to a continuous column that contains the entire complement of neural retinal cell types. Our results indicate no variability (and therefore no stochastic decisions) among RSCs regarding the cell types they generate in the neural retina.

The mandatory clonality of retinal columns raises the question of its functional implications. Remarkably, two publications have recently reported that columns of neurons in the mammalian cortex have a clonal origin that favors interconnection between siblings, establishing a link between clonal generation and coordinated function (Li et al., 2012; Yu et al., 2012). It will be exciting to address whether the clonal relation within neural iArCoSs from photoreceptors to ganglion cells has similar functional implications, as observed in the mammalian cortex.

**Symmetric and asymmetric divisions among RSCs in vivo**

The availability of genetic tools to label and lineage trace single cells has allowed us to understand several features of stem cells in their natural intact environment (Doupé et al., 2012; Mascré et al., 2012; Simons and Clevers, 2011; Snippert et al., 2010; Van Keymeulen et al., 2011). The results obtained by analyzing the clones formed by stem cells at different time points after induction challenged the text-book view on how stem cells function in vivo. The long-assumed asymmetric way of cell division, in which a stem cell divides to generate an identical daughter and a differentiated cell (or a progenitor with limited differentiation or proliferative potential), was replaced by a neutral drift model in which stem cells prefer symmetric division to generate either two stem cells or two committed progenitors (Simons and Clevers, 2011). In this way, homeostasis (and the number of stem cells per organ/tissue) is maintained at the level of the niche, although clones expand or are eliminated while competing for space and/or niche resources.

In most vertebrate organs in which symmetric divisions are the rule among stem cells, homeostasis consists in the life-long maintenance of the proper number of differentiated cells, replacing lost cells by new ones. The fish retina constitutes a different system, in which homeostasis involves organ growth by the addition of new cells: older adult retinæ have more cells than younger adult retinæ. Our data show that RSCs undergo preferentially (but not only) asymmetric cell divisions. It is important to stress that our statements about asymmetric divisions focus on the cell types that are generated after mitoses, and not on the cellular components that might be involved in making the decision. Clones that are generated during late embryogenesis are still present and growing actively several months after, showing that they do not displace each other but rather extend parallel to the growth axis. This indicates that stemness among medaka RSCs is a very stable decision that guarantees maintaining clonal progression throughout life.

Notably, RSCs still prefer asymmetric divisions during response to injuries in the transient amplifying domain. This is in agreement with the absolute penetrance of RSC multipotency: RSCs do not choose between different options but rather do the very same thing irrespective of environmental conditions. Plastic responses to injuries rely on the progenitors rather than on stem cells, as evidenced by the transient nature of expansion or reduction of the clone’s width that we observed. Along the same line, it was recently reported that both in the zebrafish and the Xenopus retina, the response to nutritional changes is mediated by RPCs and not by RSCs (Love et al., 2014). Progenitors have shorter cell cycles and therefore generate lots of cells in reduced periods of time to immediately respond to sudden cell loss. Allocating the task of a transient regenerative response to RPCs seems a superior solution to compromising RSCs, the actions of which will have a permanent impact and will be maintained during the entire life of the fish. However, we have not addressed the response of RSCs to ablations in the RSCs domain, but by definition, if they do regenerate the same cell type it can only be through symmetrical divisions. In the
absence of this experiment, we cannot comment on the divisional preference of RSCs during regeneration of their own domain.

The results that we report here regarding the life-long activity of RSCs in the mature retina are in agreement with our previous results using transplantation experiments at blastula stages. As the induction of recombination at mature stages of the retina generates iARCoSs that resemble the ArCoSs obtained via transplantation, which reflect the behavior for RSCs at earlier stages, our results suggest that the RSC pool is established and fixed from the early retina development onwards. Whether this pool of stem cells is actively separated from the RPCs involved during the first wave of differentiation during retinogenesis or whether it is just the remains of cells that were not reached by the differentiation wave is still to be demonstrated.

Our data also show that the preference for predominant asymmetric divisions is maintained by RSCs throughout life. If there were a shift to symmetric divisions at some point during late adulthood, we would expect to observe the loss of straight iARCoS and the appearance of a more stochastic scenario, with iARCoSs disappearing or overexpanding. Even in the oldest fish analyzed, the shape of ArCoS and iARCoS is maintained throughout life. The reliance on preferential asymmetric cell division constitutes a distinct feature of the adult CMZ, highlighting the behavioral spectrum of vertebrate post-embryonic stem cells and raising questions about the niche factors and molecular machinery that underpin its regulation.

MATERIALS AND METHODS

Fish stocks and generation of transgenic fish lines

Medaka (Oryzias latipes) stocks were maintained as previously described (Rembold et al., 2006a). Transgenic lines were generated using a plasmid containing I-SceI sites (pBS/I-SceI) (Rembold et al., 2006a; Thermes et al., 2002). Fish were maintained in a fish facility built according to the local animal welfare standards (Tierversuchsgesetz §11, Abs. 1, Nr. 1), and animal experiments were performed in accordance with European Union animal welfare guidelines. The facility is under the supervision of the local representative of the animal welfare agency.

Gaudi\textsuperscript{HspCre.A}
The 1.7 kb zebrafish Hsp70 promoter was cloned in a pBS/I-SceI-containing a nuclear-localized, codon optimized CRE recombinase. The plasmid pBS/I-SceI/Hsp70::Cre-NLS contains the insertional reporter cmlc2::EGFP.

Gaudi\textsuperscript{UBiq.iCre}
The 3.5 kb zebrafish ubiquitin promoter (Mosimann et al., 2011) (Addgene plasmid 27320) was cloned in a pBS/I-SceI containing a tamoxifen-inducible Cre recombinase (from pBluescript). The plasmid pBS/I-SceI/ubiquitin\textsuperscript{ERT2} contains the insertional reporter cmlc2::ECFP.

Gaudi\textsuperscript{RSG}
The 3.5 kb zebrafish ubiquitin promoter replaced Hsp70 promoter in Addgene plasmid 24334 (Hesselson et al., 2009). The plasmid pBS/I-SceI/ubiquitin::LoxP-DSRed::LoxP::H2B-EGFP contains a cry::ECFP as an insertional reporter. Gaudi\textsuperscript{RSG} transgenic fish have been successfully recombined over five generations.

Gaudi\textsuperscript{BBW2.1}
The 3.5 kb zebrafish ubiquitin promoter (Mosimann et al., 2011) was subcloned in a pBS/I-SceI containing an inverted BBW2.1 cassette (Livet et al., 2007), upstream of the Cerulean FP. Gaudi\textsuperscript{BBW2.1} transgenic fish have been successfully recombined for over six generations.

Gaudi\textsuperscript{LBBW}
A fragment containing ubiquitin::LoxP-DSRed::LoxP from Gaudi\textsuperscript{RSG} was subcloned in a pBS/I-SceI containing a BBW2.1 cassette, upstream of the Cerulean FP. Gaudi\textsuperscript{LBBW} transgenic fish have been successfully recombined for over three generations.

LoxP\textsuperscript{OUT}
Gaudi\textsuperscript{RSG} were outcrossed to Gaudi\textsuperscript{HspCre.A} and fish were heat-shocked and grown to adulthood. A female Gaudi\textsuperscript{RSG}, Gaudi\textsuperscript{HspCre.A} produced embryos that were entirely green, irrespective of the Hsp70::Cre-NLS insertion and heat-shock treatment, indicating that recombination of the LoxP cassette happened in the germline of the mother and transmitted to the progeny. LoxP\textsuperscript{OUT} fish have been successfully maintained for over four generations.

Injuries in the retina

Animals at 3 weeks post-hatching were anesthetized in 0.5× Tricaine (A5040, Sigma-Aldrich). Under microscopic visualization, the right retina was stabbed in the CMZ region in the dorsal and the two lateral quadrants with a glass needle (0.1 mm diameter). Only the tip of the needle was inserted to avoid injuring cells behind the CMZ. After treatment, the fish were returned to their tanks to recover. Left retinae were used as controls.

Inducible expression of Cre recombinase

To induce CRE recombinase expression in Gaudi\textsuperscript{HspCre.A} juveniles were kept at 25°C for 3 h and transferred to ERM at 42°C. Gaudi\textsuperscript{UBiq.iCre} juveniles were treated with a 2.5 μM tamoxifen (T5648 Sigma) solution for 3-24 h and washed afterwards with fish water. To favor sparse recombination of the Gaudi\textsuperscript{RSG} line in the CMZ, we followed the same procedure using ERM at 36°C (for Gaudi\textsuperscript{HspCre.A} and 30-60 min of 2.5 μM tamoxifen (for Gaudi\textsuperscript{UBiq.iCre}).

Transplantation experiments

Transplantations were carried out as previously described (Ho and Kane, 1990; Rembold et al., 2006b). Ten to 15 Wimbledon\textsuperscript{+/−} blastula cells were transplanted into wild-type blastulae. Transplanted embryos were kept in 1× ERM supplemented with antibiotics (penicillin-streptomycin solution from Sigma, P0781, 1/200) and selected for EGF\textsuperscript{+} cells.

Antibodies and staining

Primary antibodies used in this study were rabbit anti-GFP (Invitrogen, A-11122, 1/750), chicken anti-GFP (Invitrogen, A-10262, 1/1000), rabbit α-DsRed (Clontech, 632496, 1/750), mouse anti-BrdU/IdU (Becton Dickinson, 347580, 1/25), rabbit anti-PKCα (Santa Cruz, sc-208 1/400), mouse anti-PCNA (Santa Cruz, sc-56, 1/400), rabbit anti-phospho-histone 3 (Upstate, 06-570, 1/500), mouse anti-HuC (Invitrogen, A-21271, 1/200), mouse anti-GS (BD Biosciences, 610517, 1/500), mouse anti-parvalbumin (Chemicon, MAB1572, 1/400) (Inoue and Wittbrodt, 2011). Secondary antibodies were Alexa488 anti-rabbit and Alexa546 anti-mouse (Invitrogen, A-11034 and A-11030 respectively, 1/400), and DyLight488 anti-chicken, DyLight549 anti-rabbit and Cy5 anti-mouse (Jackson, 703-485-155, 112-505-144 and 715-175-151, respectively, 1/400).

Imaging

Samples were imaged using an Olympus MVX10 binocular coupled to a Leica DFC500 camera (living juveniles, entire retina), a Nikon AZ100 coupled to a Nikon C1 (entire retinae), and a Leica TCS SP5.

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Competing interests

The authors declare no competing financial interests.

Author contributions

L.C. and J.W. conceived the project, designed the experiments and wrote the manuscript. L.C., J.-J.A., B.H., K.L., T.K., I.K., C.U. and E.H. performed the experiments. L.C., W.A.H., B.D.S. and J.W. analyzed and discussed the data, and edited the manuscript.
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Supplementary material
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