Neural stem cell isolation from the whole mouse brain using the novel FABP7-binding fluorescent dye, CDr3

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Abstract

Methods for the isolation of live neural stem cells from the brain are limited due to the lack of well-defined cell surface markers and tools to detect intracellular markers. To date most methods depend on the labelling of extracellular markers using antibodies, with intracellular markers remaining inaccessible in live cells. Using a novel intracellular protein FABP7 (Fatty Acid Binding Protein -7) selective fluorescent chemical probe CDr3, we have successfully isolated high FABP7 expressing cells from the embryonic and adult mouse brains. These cells are capable of forming neurospheres in culture, express neural stem cell marker genes and differentiate into neurons, astrocytes and oligodendrocytes. Characterization of cells sorted with Aldefluor or antibodies against CD133 or SSEA-1 showed that the cells isolated by CDr3 exhibit a phenotype distinct from the cells sorted with conventional methods. FABP7 labelling with CDr3 represents a novel method for rapid isolation of neural stem cells based on the expression of a single intracellular marker.

Keywords: FACs, BLBP, neurosphere, self-renewal, small molecule fluorescent probe

Introduction

Neural stem cells (NSCs) in the mammalian brain are characterized by their ability to self-renew and differentiate into multiple types of neuron and glia. They form free floating spheres if cultured in a medium containing growth factors (Fischer et al., 2011). These properties make them much coveted tools in the field of regenerative medicine where it is hoped they can be utilized to regenerate neurons that have been lost as a result of disease or injury (Ruff and Fehlings, 2010). NSCs are most abundant in the developing foetal brain and persist throughout whole life generating new neurons and glia continuously. The study of NSCs from adult brain tissue is particularly challenging because the cells are low in number (0.1-1%) and require specialized and tedious cell preparation techniques which have limited yield (Brewer et al., 2007; Galli et al., 2003). As such, considerable research has been focused on developing isolation techniques for these elusive cells.

Major obstacles to isolate NSCs have been the lack of NSC specific cell surface markers and tools to detect intracellular markers which are more specific both in the developing and mature brain. Commonly used methods of NSC labelling either involve antibody for cell surface marker labelling or fluorescent protein expression with the desired marker of interest (Fischer et al., 2011; Corti et al., 2007; Coskun et al., 2008). Presently, the most widely used cell surface markers for the isolation of NSCs are CD133 and SSEA-1 (Capela and Temple, 2002; Corti et al., 2007). The fluorescent dye, AldeFluor which is catalyzed by the intracellular metabolic enzyme aldehyde dehydrogenase to be retained in the cell has also been demonstrated to enrich for NSCs (Corti et al., 2006). More recently, the stem cell specific fluorescent dye CDy1, developed by us as a pluripotent stem cell probe (Im et al., 2010) has also been shown to be able to isolate NSC from the mouse brain although the mechanism of action remains to be elucidated (Vukovic et al., 2013). Using a combination of the three NSC markers (CD133, SSEA-1 and AldeFluor), Obermair et al. reported several

subpopulations of neural stem/progenitors within the brain (Obermair et al., 2010). Fischer et al. reported the isolation of NSCs from GFAP+/CD133+ cells from the adult mouse brain (Fischer et al., 2011). On the other hand, other studies have shown that the expression of CD133 in NSC depends on developmental stage (Pfenninger et al., 2007) and that a significant portion of NSC do not express this marker (Sun et al., 2009).

Brain cell preparation necessitates the use of enzymatic dissociation to mitigate the otherwise damaging effects of mechanic dissociation, with trypsin and papain being the two most commonly used agents (Oliver-De la Cruz and Ayuso-Sacido, 2013). Inconsistent findings in multiple independent studies might have been attributed to cell surface antigen cleavage during enzymatic dissociation of brain tissue (Panchision et al., 2007). Efforts have been made to minimise antigen cleavage by developing milder enzymatic treatment agents. However their effectiveness is subject to the antigen of interest and it still difficult to eliminate the possibility of antigen cleavage completely. Isolation of live NSC by labelling intracellular markers with antibodies is not possible, because it requires membrane permeabilization which kills the cells thus making it unfeasible for self renewal and other live cell assays downstream of labelling. Fluorescent protein co-expression with an intracellular marker requires tedious methods to generate transgenic mice and must be approached with caution since genetic modification may affect the native cellular physiology. Therefore, direct labelling of a specific intracellular marker would provide a means of simple and effective isolation of NSC for downstream applications.

In a previous study we presented a small molecule fluorescent probe, compound of designation red 3 (CDr3), which binds specifically to FABP7 in NSCs of both human and mouse origin (Yun et al., 2012). Due to its low molecular weight (Mr = 572) and high lipophilicity (cLogP = 8), CDr3 is capable of diffusing passively through the cell membrane and is retained by FABP7-expressing NSCs leading to selective staining. FABP7 is expressed

ubiquitously in developing radial glia in the central nervous system, with different subpopulations of progenitor cells restricted to individual fates (Schmid et al., 2006). At late embryonic and post-natal stages, a subpopulation of these radial glia give rise to NSCs that are implicated in adult neurogenesis (Malatesta et al., 2008). NSCs express high level of FABP7. However, its expression level drops significantly by differentiation of NSCs. (Conti et al., 2005; Arai et al., 2005). Studies have also shown that NSC numbers are dramatically reduced in FABP7 knockout mouse models (Matsumata et al,. 2012). This strongly suggests that FABP7 plays a critical functional role in neurogenesis and regeneration (Matsumata et al., 2012). Both the embryonic and adult mouse brains are known to contain populations of cells that express varying levels of FABP7 (Gerstner et al., 2008). Here we demonstrate that CDr3 can be used to isolate high FABP7 expressing live cells from the embryonic and adult mouse brains which share characteristics with known NSC markers. CDr3 can be reliably used for the easy and rapid isolation of NSCs from complex brain tissues.

Materials and Methods

Brain cell preparation

For the preparation of cells from the embryonic and adult mouse brains, E14 embryos and brains from 8-12 week old adults were used. For embryos, the whole embryo brain was dissected out and digested in 0.25% trypsin-EDTA (GIBCO) for 15 minutes at 37 ºC. To achieve a single cell suspension, the trypsin was neutralized with FBS and the brains were resuspended in PBS before sequential trituration using pipettes of decreasing diameter. Cell suspensions were washed three times in PBS before filtering through a 40 µm strainer. For the adult brain, whole brain without cerebellum was dissected and minced in 20U/mg of papain solution (Worthington) for 1 hour at 37 ºC before trituration and washing as described above. Animals were prepared and sacrificed in accordance with the protocol approved by the Institutional Animal Care and Use Committee.

Cell sorting and flow cytometry

Embryonic and adult brain cells were stained for 1 hour with 500 nM of CDr3 in neurosphere growth media consisting of DMEM/F12, B27 without vitamin A, bFGF (10 ng/ml) and EGF (20 ng/ml) (GIBCO) and penicillin-streptomycin glutamine before sorting using the MoFlo XDP (Beckman Coulter). For AldeFluor staining, we used the AldeFluor^{TM} kit (STEMCELL Technologies) according to the manufacturer's instructions. Labelled single cell suspensions were pre-gated for size and granularity before sorting based on their relevant fluorescence marker. For immunostaining and sorting, cells were stained with CD133 (Biolegend, 1:250) and SSEA-1 (Millipore, 1:250) antibodies for 1 hour followed by secondary antibody conjugated to Alexa Fluor 488 (Life Technologies). For flow cytometry analysis, the primary antibodies against CD133 (Biolegend), SSEA-1 (Millipore), FABP7 (Abcam) and ALDH1A1

(Santa Cruz) were diluted 1:500 to stain. Flow cytometry analysis was carried out on a BD LSRII analyzer. All sorting experiments were carried out n =3-5 times with the data shown reflecting the data from one representative independent experiment.

Neurosphere assay and differentiation

Embryo and adult derived neurospheres were grown in DMEM/F12 supplemented with B27 without vitamin A, 20 ng/ml of EGF, 10 ng/ml of bFGF and penicillin-streptomycin glutamine and cultured for 4-5 days (embryo) or 5-7 days (adult). For neurosphere assay cell sorting, a total of 10,000 cells were plated per well of a six well plate in neurosphere growth media. Samples were plated in triplicate and the neurospheres were counted 4-5 days (embryo) or 5-7 days (adult) later. To assess differentiation capacity, neurospheres were differentiated on poly-D-lysine (Sigma) coated plates for 5 days in DMEM/F12 + 10% FBS (GIBCO) before fixation in 4% paraformaldehyde. To determine self-renewal for secondary, tertiary and further assays, primary assay neurospheres were pooled and triturated to a single cell suspension in 0.05 M NaOH followed by neutralization with 0.05 M HCl. The cells were then re-plated at a density of 1000-2000 cells per well in fresh neurosphere growth media.

MTS assay

For the measurement of cell proliferation by MTS assay (Promega), 100,000 cells were sorted with their respective markers (whole, CDr3, AldeFluor, CD133 or SSEA-1) and re-cultured for 5-6 days to form neurospheres. Spheres were subsequently dissociated using 0.05 M NaOH treatment and plated in entirety in triplicate on a 96 well plate. The addition of 20 µl of MTS reagent was added to the cell solution and the readings were measured at 490 nm after 4 hours incubation.

Cell immunostaining

For triple immunostaining of neurons, astrocytes and oligodendrocytes from differentiated neurospheres, cells fixed with 4% paraformaldehyde were first blocked in 1% BSA followed by O4 antibody staining (Millipore, 1:1,000) and secondary antibody staining (AlexaFluor 488, Life technologies). This was followed by permeabilization with 0.1% Triton-X and antibody staining with β-III-tubulin (Sigma) and GFAP (Wako) at 1:500-1:1,000 dilutions. For further secondary antibody staining, AlexaFluor 546 and Cy5 conjugated antibodies (Life Technologies) were used respectively at dilution factors of 1:100. Images were acquired on the Nikon Ti microscope at 100X magnification.

Gene expression analysis

For the analysis of gene expression, RNA was extracted from whole, bright and dim FACS sorted cells using the QIAGEN RNeasy purification kit according to the manufacturer's instructions. RNA samples were analyzed by qRT-PCR with the SYBR Green Master mix reagents (Applied Biosystems) with normalization to GAPDH expression. The full list of primers and their respective efficiencies can be viewed in the Supplementary Materials and Methods.

Results

CDr3 can be used to isolate FABP7-positive cells from the embryonic and adult mouse brains

FABP7 is expressed in a large population of cells in the developing embryo brain but only constitutes about 1% of the brain cell population by adult hood (Brewer et al., 2007). CDr3 (Figure 1a) staining could be visualized in culture where certain cells in neurospheres and dissociated brain cells appeared much more brightly stained than their surrounding cells (Figure 1b). When applied to mixtures of primary embryonic and adult mouse brain cell preparations, CDr3 was sufficiently specific for the clear identification of a distinct population of primary brain cells (Supplementary Figure 1). As such we were interested to determine if high FABP7 expression alone (as gated by the brightest 10% of CDr3 positive cells – Supplementary Figure 1) was sufficient for the isolation and characterization of NSCs from the brain.

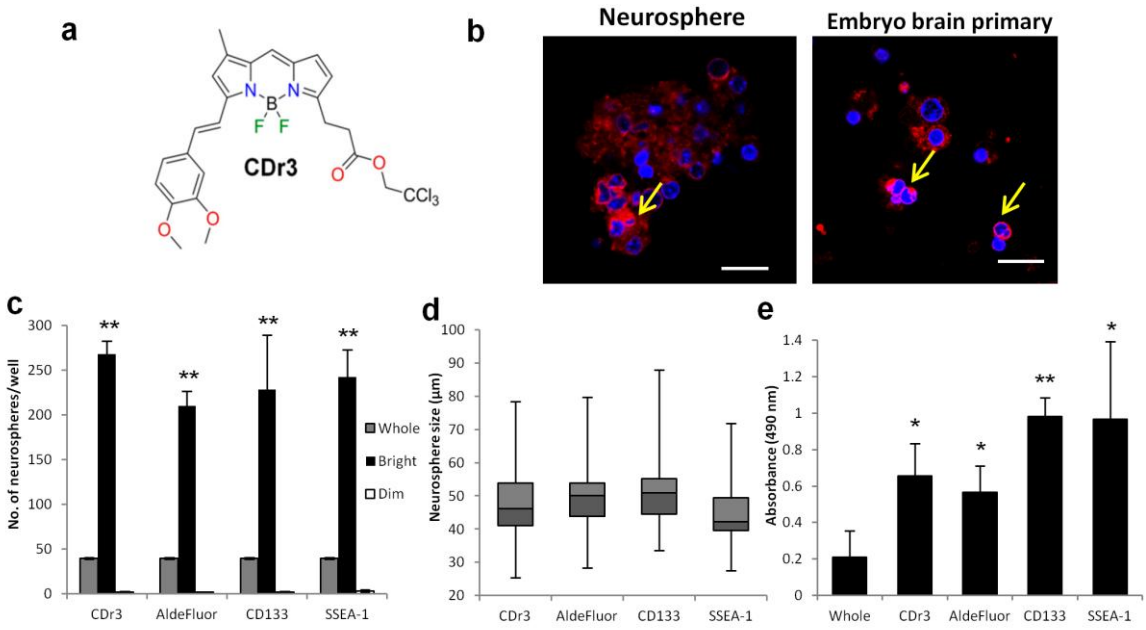

Figure 1. *(a) Structure of the Fabp7 selective fluorescent dye, CDr3. (b) Confocal microscope images of CDr3 stained neurosphere and embryo brain primary cells. The yellow arrow indicates CDr3^{Bright} cells. Scale bar, 20 µm. (c) Number of neurospheres generated from unsorted (whole) and sorted embryo brain cells by CDr3, AldeFluor, CD133 antibody*

*and SSEA-1 antibody. The sorting was carried out n=5 times with the data reflecting the best yield from one independent experiment. An unpaired two tailed t test was performed (whole vs. bright, **p<0.01). (d) Size of neurospheres generated from sorted embryo brain cells by CDr3, Aldefluor, CD133 antibody and SSEA-1 antibody. (e) An MTS assay was performed on cell dissociated from unsorted (whole), CDr3, AldeFluor, CD133 and SSEA-1 sorted neurospheres. An unpaired, two tailed t test was performed (whole vs. sorted, *p<0.05, **p<0.01). Values shown represent mean ±S.D.*

High FABP7 expressing cells form neurospheres

For assessment of self-renewal capacity, the brightest and dimmest 10% of the CDr3 positive cell populations from E14 embryonic mouse brains were sorted out and plated for neurosphere assay. $CDr3^{Bright}$ cells were highly proliferative compared to their $CDr3^{Dim}$ counterparts, which hardly formed spheres (Figure 1c).

When compared to the unsorted (whole) fraction, CDr3 NSC enrichment resulted in an up to 5 fold increase in neurosphere yield which was comparable to established methods of neurosphere enrichment by cell sorting using AldeFluor or antibodies against CD133 and SSEA-1 in number (Figure 1c) and size (Figure 1d) of neurospheres when applied to embryonic mouse brain cells. Because neurospheres themselves cannot be considered clonal entities (Singec et al., 2006), an MTS assay was also carried out to measure the proliferative activity of the isolated cells (Figure 1e). All marker sorted cells were significantly more proliferative compared to the whole sorted fraction.

High capacity for self-renewal is characteristic of NSCs as opposed to neural progenitors, which lose their self-renewal capacity after several passages. CDr3 sorted NSC from the embryo and adult brain were successfully serial passaged at a yield that was higher than that of neurospheres generated from unsorted cells (Supplementary Figure 2a and b). A comparison of neurospheres grown in 500 nM of CDr3 and 0.1% DMSO containing media showed that the dye does not affect the ability of the cells to form spheres (Supplementary Figure 2c and d).

CDr3 enriches for NSCs in the adult brain

Adult NSCs are far fewer and have more restricted growth conditions as compared to embryo derived cells (Morshead et al., 2004). In adult brain cell preparation, there is also considerable amount of myelin and cell debris which makes cell isolation difficult (Figure 2a). The sub ventricular zone (SVZ) of the adult brain is known to harbour the highest percentage of NSCs and is thus usually the region of choice when isolating adult NSCs to reduce complexity of the tissue. We compared the number of primary neurospheres generated from a whole hemisphere versus the dissected SVZ region of the other hemisphere from the same brain. Our data showed that the SVZ accounts for up to 50% of the neurospheres formed from one hemisphere of the brain. However, a significant portion of neurospheres can also be derived from the remaining regions of the brain. Thus by dissecting only the SVZ, up to 50% of neurosphere yield from the brain is lost which is considerable given the relative scarcity of NSCs in the adult brain. CDr3 enrichment provided a much cleaner cell preparation of adult brain derived neurospheres which were bigger and could be used for further experiments (Figure 2a and b). Compared to AldeFluor, CDr3 sorting generated higher levels of enrichment (Figure 2c). Attempts to sort cells from the whole adult brain by CD133 and SSEA-1 antibody sorting failed to produce any spheres in culture.

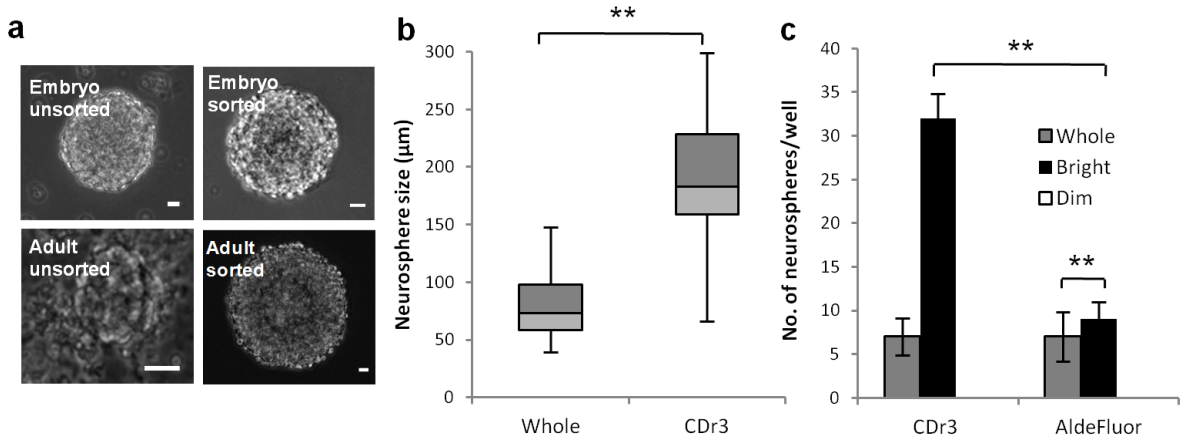

Figure 2. *(a) Neurospheres generated from unsorted and sorted cells isolated from embryo and adult brains. Scale bar, 20 µm. (b) Size of neurospheres generated from unsorted (whole) and sorted adult mouse brain cells by CDr3. A Mann-Whitney U test was performed*

*where **p<0.01. (c) Number of neurospheres generated from unsorted (whole) and sorted adult mouse brain cells by CDr3 and Aldefluor. CDr3 yielded a significantly higher number of neurospheres (**p<0.01) compared to Aldefluor. Values shown represent mean ±S.D.*

CDr3 isolated neurospheres are multipotent

NSCs are capable of differentiating into all three neural cell types i.e. neurons, astrocytes and oligodendrocytes, whereas neural progenitors have a more restricted differentiation lineage (Klein et al., 2004). Upon differentiation of neurosphere derived NSCs, FABP7 expression is decreased and CDr3 staining is lost (Supplementary Figure 3a). CDr3 isolated neurospheres from both embryonic and adult brains could be effectively differentiated into all three cell types (Figure 3a), with robust staining for their respective antibody markers. This potential for multi-lineage differentiation validates their NSC phenotype.

To demonstrate that CDr3 does not affect the ability of neural stem cells to differentiate into neurons which would potentially impede neurogenesis, neurospheres were dissociated into single cells and induced to neuronal differentiation in the presence and absence of CDr3. There was no significant difference in the number of neurons generated from DMSO and CDr3 treated cells (Supplementary Figure 3b).

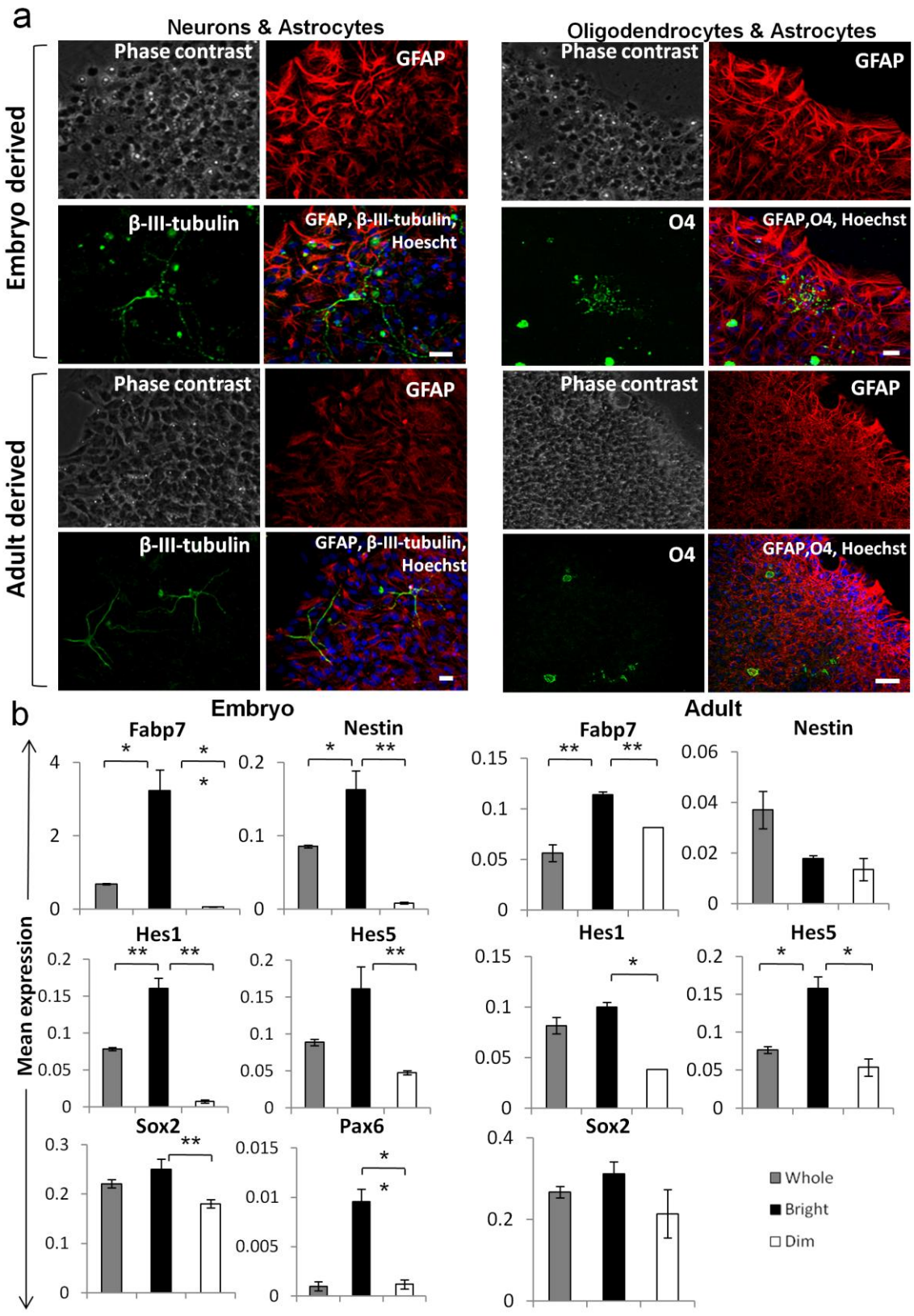

Figure 3. *(a) CDr3 sorted primary neurospheres from the E14 embryo and adult brains were differentiated and immunostained for β-III-tubulin (neurons), GFAP (astrocytes) and O4 (oligodendrocytes). Scale bar, 20 µm. (b) Expression of neural stem cell associated genes in unsorted (whole) and sorted embryo and adult brain cells by CDr3. An unpaired two tailed t*

test was performed for all genes (p<0.05 and **p<0.01). Values shown represent mean ±S.D.*

High NSC gene expression in CDr3^{Bright} cells

In addition to FABP7, there are numerous other gene markers that are known to be distinctively expressed in NSCs (Fischer et al., 2011; Corti et al., 2007; Corti et al., 2006). For the gene expression analysis, we used 8 different gene markers for analysis of the cell populations isolated from the embryo and adult brains. CDr3^{Bright} cells were found to have higher expression of FABP7 compared to CDr3^{Dim} cells in both embryo and adult sorted cells. This is consistent with the dye's FABP7 selectivity (Figure 3b). In addition, CDr3^{Bright} cells also showed high expression of other known NSC markers such as Nestin, Hes1 and Hes5. Pax6 expression was too low to be detected in the adult brain. Comparison of unsorted (whole) versus CDr3^{Bright} samples also showed that CDr3 sorting significantly enriched for high FABP7 expressing cells up to from 2-20 fold compared to unsorted embryo derived brain cells (Figure 3b). This confirms that CDr3 is effective for use as a sorting tool for the isolation of NSCs from the embryonic and adult brains.

NSC phenotypes depend on isolation markers

Due to its intracellular localization, FABP7 is seldom used for cell isolation. With the development of CDr3 as a live NSC probe, we are now able to characterize these cells based on their level of FABP7 expression alongside other cell surface markers such as CD133 and SSEA-1. It is interesting to note that while the fluorescent probe, AldeFluor has been successfully employed for the use of NSC isolation (Corti et al., 2006), its representative binding target – ALDH1A1 is not generally known to be a marker for NSCs. Moreover,

recent studies have shown that this protein may in fact be dispensable for stem cell function (Levi et al., 2009).

To characterize the brain cells by their respective phenotypes, cells derived from the whole E14 embryo and adult mouse brains were stained and sorted by CDr3, SSEA-1, CD133 or AldeFluor. Live cells collected by sorting were then co-labelled with another NSC marker (CDr3, AldeFluor, CD133 or SSEA-1) and analyzed by flow cytometry to determine the percentage of cells expressing each NSC marker. It was observed that marker profiles at E14 and adult stage were distinct. CD133 and SSEA-1 occupied relatively selective niches at E14 but their expressions expanded to encompass a greater proportion of other cells by adult stage (Figure 4a. Supplementary Table 1). Although CDr3 was not the most restricted lineage at E14 stage, expression area was markedly reduced in the adult, especially in the proportion of CD133 positive cells. This is consistent with published literature and our own results (Supplementary Figure 4) which demonstrate that CD133 expression is decreased in the adult versus embryo stage. This suggests that CD133 may no longer be a strong NSC marker in the adult mouse brain. AldeFluor appeared to be the widest marker, with little change from E14 to adult (Figure 4a, Supplementary Figure 4). This suggests that while it is capable of isolating NSCs, it may not be a specific NSC marker.

Embryo brain cells were also sorted using AldeFluor or CD133 antibody and analyzed for FABP7 and ALDH1A1 gene expression. While both AldeFluor and CD133 positive cells in the embryo brain expressed high levels of FABP7, CD133 positive cells were found to express lower levels of ALDH1A1 than negative cells (Supplementary Figure 5a and b). For live sorted cells, $CDr3^{Bright}$ cells showed high CD133 expression (Figure 4a) but low AldeFluor staining (Figure 4b). An analysis of $CDr3^{Bright}$ embryo brain cells immunostained with ALDH1A1 antibody showed that ALDH1A1 positive cells only constitute about 10-20% of CDr3 bright sorted cells (Figure 4c).

To further assess the importance of each marker in conjunction with one another, subpopulations of cells were sorted from E14 brains and assessed for neurosphere forming capacity. SSEA-1^{+}/AldeFluor $^{-}$ cells formed the largest number of spheres (150 spheres), SSEA-1^{+}/$CD133^{-}$ cells formed few spheres (37 spheres) (Supplementary Table 2). This underscores the importance of both CD133 and SSEA-1 as NSC markers in the developing brain. However attempts to sort cells based on $CD133^{+}$ or SSEA-1^{+} alone did not produce the maximal number of spheres (45 spheres from $CD133^{+}$/SSEA-1^{-}, 37 spheres from SSEA-1^{+}/$CD133^{-}$). The inclusion of CDr3 as a sorting marker to SSEA-1+ or CD133+ cells restored neurosphere yield (45 spheres from $CD133^{+}$/SSEA-1^{-} to 114 spheres from $CD133^{+}$/$CDr3^{Bright}$, 37 spheres from SSEA-1^{+}/$CD133^{-}$ to 94 spheres from SSEA-1^{+}/$CDr3^{Bright}$. However the inclusion or exclusion of AldeFluor as a sorting marker to SSEA-1+ and CD133+ did not appear to influence sphere yield significantly. Including CD133 alongside CDr3 improved sphere yield by up to 10 times. These results reflect the heterogeneity of NSCs.

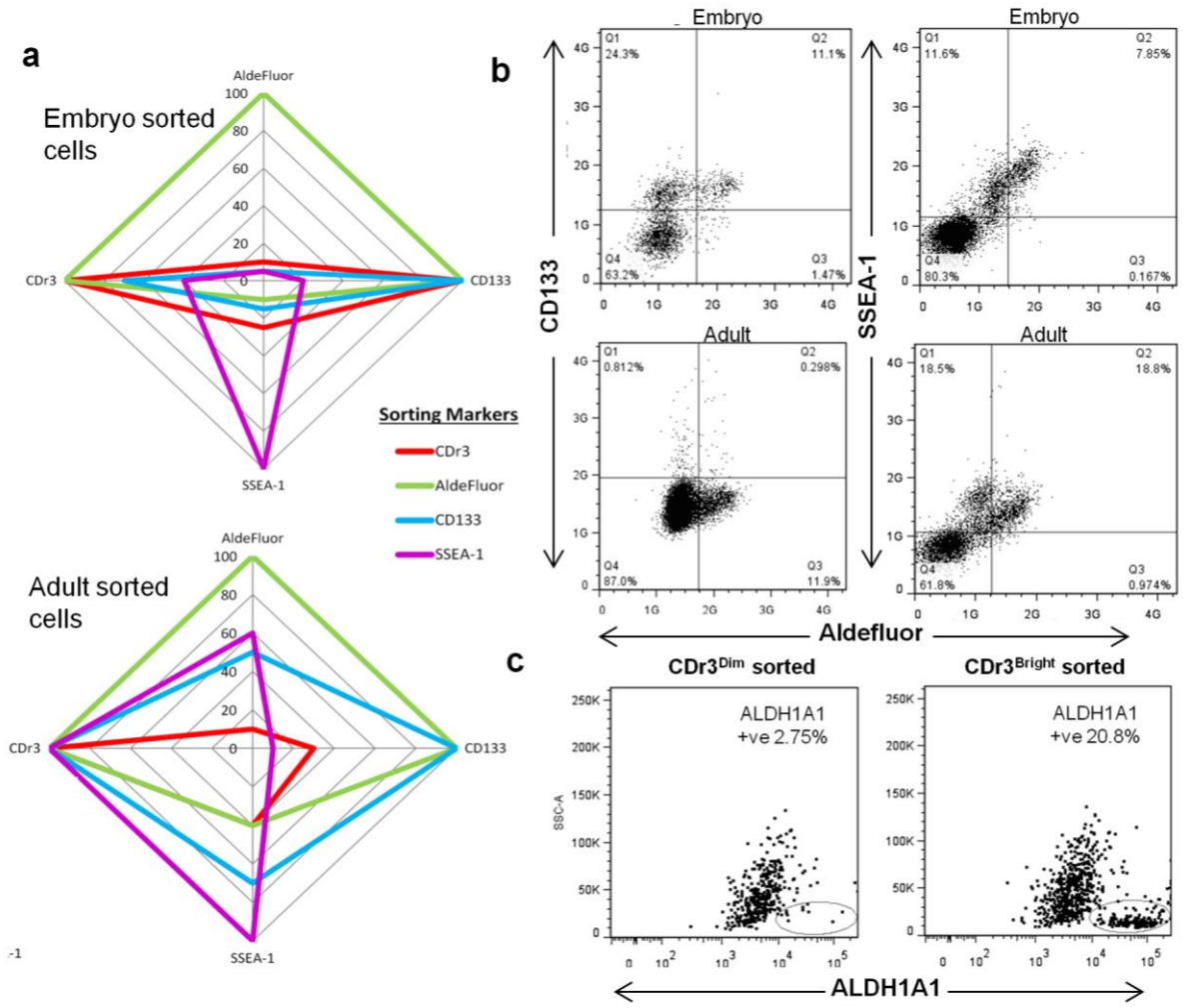

Figure 4. *(a) The percentage of marker sorted cells from embryo brain or adult brain expressing other markers measured by flow cytometry. The live cells were co-labelled with CDr3, AldeFluor, CD133 antibody and SSEA-1 antibody and plotted on a radar plot to illustrate the spread of marker expression when each population was limited to sorting by a particular marker as well as the respective expression changes from embryo to adult. (b) The percentage of CDr3^{Bright} cells co-labelled by AldeFluor, CD133 antibody and SSEA-1 antibody. (c) ALDH1A1 expressions in CDr3^{Bright} cells were assessed by flow cytometry of immunostained cells.*

Discussion

While FABP7 has been shown to be expressed in NSCs, it has not been used for live NSC isolation from a mixture of various types of cells largely because of its intracellular nature which necessitates cell permeabilization and hence cell death in the process of detection using antibodies. In this study, we demonstrated that the availability of CDr3 as an intracellular live NSC probe now makes it possible to isolate NSCs by the expression of FABP7.

To date, CD133 remains the most established NSC marker for immunoseparation of NSCs from brain tissue (Fischer et al., 2011; Corti et al., 2007). The importance of CD133 is highlighted in the fact that almost all cells positive for other markers show strong CD133 expression and that the inclusion or exclusion of CD133 as a marker from CDr3 positive populations strongly influences sphere yield. Our data has shown that CD133 expression in the embryonic brain is generally high (Figure 4, Supplementary Figure 4). But little CD133 expression was detected in the adult brain which is in agreement with data reported by Pfenninger et.al (Pfenninger et al., 2007). Interestingly, the proportion of CD133 expressing cells within the CDr3 population fell from embryo to adult but expanded across other markers. This represents a drawback of the use of CD133 as a NSC marker in the adult brain and suggests that its role as a NSC marker differs depending on the age.

AldeFluor is a small molecule dye which has been shown to be able to isolate NSCs from the brain (Corti et al., 2006). Compared to CDr3, it requires more preparation such as dye activation and blocking of the ABC transporter (Marcato et al. 2011). Its binding target in stem cells has been shown to be aldehyde dehydrogenase (ALDH1A1) (Storms et al., 1999). However only 10% of high FABP7 expressing cells were ALDH1A1 positive and its expression appeared to be broad ranging in both the embryo and the adult. The higher percentage of ALDH1A1 expressing cells in the dim fraction of CD133 sorted cells also

suggests that that the ALDH1A1 positive population is underrepresented or absent in the CDr3 positive cell fraction and that the three markers (FABP7, CD133 and ALDH1A1) may not be co-expressed in the major population of NSCs. Neurosphere yields from AldeFluor sorted adult brain cells were also lower than that from CDr3 sorting, and the inclusion of Aldefluor as a subpopulation marker did not affect neurosphere yield significantly. This suggests that while some NSCs may express elevated levels of ALDH1A1, this expression is not a critical marker of NSC property. This could largely in part be due to the presence of alternative ALDH isoforms present in mouse NSCs (Marcato et al., 2011).

It was also observed that the expression of Nestin, Hes1 and Sox2 in CDr3^{Bright} adult sorted cells were lower than the unsorted fraction or largely unchanged. Studies have shown that it is likely that multiple classes of neural stem/progenitor cells exist, each classified by distinct gene expression profiles. A study by Hendrickson et al., 2011 has found that four distinct classes of nestin expressing cells exist in the mature adult brain, one of which consists of a class of terminally differentiated neurons (Hendrickson et al., 2011). This suggests that the expression of nestin alone may not be a reliable indicator of neural cell stemness in the adult brain and may account for the lack of nestin enrichment in CDr3^{Bright} sorted adult brain cells. In another study by Hutton and Pevny, the authors have also found that Sox2 is differentially expressed between neural progenitors and intermediate progenitor populations (Hutton and Pevny, 2011). In the study by Obermair et al. (Obermair et al., 2010), Sox2 expression in CD133, SSEA-1 or ALDH positive cells isolated from adult mouse brain subventricular zone is lower than in the marker-negative cells. And even between CD133 positive cells, Sox2 expression level dramatically varies depending on the region where the cells are isolated. This suggests high Fabp7 expressing neural stem cells may represent an alternative class of adult neural stem cells that retain neural stemness while expressing a unique neural stem cell gene profile.

NSC isolation from the adult mouse brain is more challenging given the vast amount of white matter and glia packaging the brain. Most NSC isolation protocols require additional purification steps prior to NSC sorting such as SVZ dissection or density centrifugation to remove myelin debris (Fischer et al., 2011; Brewer et al., 2007; Capela and Temple, 2002). In the case of CDr3, we were interested to show if the compound was specific enough to isolate NSCs without the need for additional purification steps. When NSCs were isolated from the whole adult brain using the different methods and cultured for neurosphere assay, the largest number of neurospheres was generated from the cells isolated by CDr3, while no spheres were formed from CD133 and SSEA-1 antibody sorted cells. Failure of these antibody-based isolation methods is likely due to lower expression of these genes in adulthood (Pfenninger et al., 2007). In the absence of a single defined NSC marker, several research groups have attempted to classify their observations into different sub populations of NSCs based on marker expression and differentiation potential. Obermair et al. (Obermair et al., 2010) reported that only SSEA-1^{+}/ALDH1^{+} cells are tripotent among the three main sub populations of NSCs they identified: SSEA-1^{+}, ALDH1^{+} and SSEA-1^{+}/ALDH1^{+}. Interestingly, rapidly dividing cells in the SVZ were found to be CD133^{-}/SSEA1^{-}/ALDH1^{-} whereas CD133^{+} formed a very slowly dividing subtype of cells. Given our current findings, NSCs may be best described as being predominantly FABP7^{+}/SSEA1^{+}/CD133^{+}/ALDH1^{-}, with a second predominant subset being FABP7^{+}/SSEA-1^{+}/CD133^{+}/ALDH1^{+}.

The heterogeneity of marker expression in these isolated cells and its changes over development suggest that a more comprehensive characterization of these cells is required for an understanding of their regenerative potential, for which the inclusion of additional intracellular isolation markers would be useful.

Conclusion

We have demonstrated that CDr3 can be reliably used as a marker for the isolation of NSCs from whole embryonic and adult brain tissue based on specific binding to the intracellular protein, FABP7. This is unique because most existing NSC isolation methods utilize extracellular markers. The inclusion of FABP7 as an additional isolation marker may shed more light on the characteristics of the NSC phenotype, thus paving the way for more efficient cell isolation techniques and characterization studies.

Acknowledgements

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Disclosure of potential conflicts of interest

C.L., D.Z., S.-W.Y and Y.-T.C. are the inventors of **CDr3** for which a patent has been filed.

References

Arai, Y., Funatsu, N., Numayam-Tsuruta, N., Nakmura, S,,Osumi, N., 2005. Role of Fabp7, a downstream gene of Pax6, in the maintenance of neuroepithelial cells during early embryonic development of the rat cortex. J. Neurosci. 25, 9752-9761.

Brewer, G.J., Torricelli, J.R., 2007. Isolation and culture of adult neurons and neurospheres. Nat. Protoc. 2, 1490-1498.

Capela, A., Temple, A., 2002. LeX/ssea-1 is expressed by adult mouse CNS stem cells, identifying them as nonependymal. Neuron 35, 865–875.

Conti, L., Pollard, S.M., Gorba, T., Reitano, E., Toselli, M., Biella, G., Sun, Y., Sanzone, S., Ying, Q.L., Cattaneo, E., Smith, A., 2005. Niche-independent symmetrical self-renewal of a mammalian tissue stem cell**.** PLoS Biol. 9, e283.

Corti, S., Nizzardo, M., Nardini, M., Donadoni, C., Locatelli, F., Papadimitriou, D., Salani, S., Del, B.R., Ghezzi, S., Strazzer, S., Bresolin, N., Comi, G.P., 2007. Isolation and characterization of murine neural stem/progenitor cells based on Prominin-1 expression. Exp. Neurol. 205, 547-562.

Corti, S., Locatelli, F., Papadimitriou, D., Salani, S., Del Bo, R., Strazzer, S., Bresolin, N., Comi, G.P., 2006. Identification of a primitive brain-derived neural stem cell population based on aldehyde dehydrogenase activity. Stem Cells 24, 975-985.

Coskun, V., Wu, H., Blanchi, B.**,** Tsao, S., Kim, K., Zhao, J., Biancotti, J.C., Hutnick, L., Krueger, R.C., Fan, G., De Vellis, J., Sun. Y.E., 2008. CD133+ neural stem cells in the ependyma mammalian postnatal forebrain. Proc. Natl. Acad. Sci. USA 105, 1026–1031.

Fischer, J., Beckervordersandforth, R., Tripathi, P., Steiner-Mezzadri, A., Ninkovic, J., Götz, M., 2011. Prospective isolation of adult neural stem cells from the mouse subependymal zone. Nat. Protoc. 6, 1981-1989.

Galli, R., Gritti, A., Bonfanti, L., Vescovi, A.L., 2003. Adult neural stem cells. Circ. Res. 92, 598-608.

Gerstner, J.R., Bremer, Q.Z., Vander Heyden, W.M., LaVaute, T.M., Yin, J.C., Landry, C.F., 2008. Fabp7 is diurnally regulated in astrocytes and hippocampal granule cells precursors in adult rodent brain. PLoS ONE 3(2), e1631.

Hendrickson, M.L., Rao, A.J., Demerdash, O.M.A., Kalil, R.E., 2011. Expression of Nestin by Neural Cells in the Adult Rat and Human Brain. PLoS ONE 6(4), e18535.

Hutton, S.R., Pevny L. H., 2011. SOX2 expression levels distinguish between neural progenitor populations of the developing dorsal telencephalon. Dev. Biol., 352, 40-47.

Im, C.N., Kang, N.Y., Ha, H.H., Bi, X., Lee, J.J., Park, S.J., Lee, S.Y., Vendrell, M., Kim, Y.K., Lee, J.S., Li, J., Ahn, Y,H., Feng, B., Ng, H.H., Yun, S.W., Chang, Y.T., 2010. A fluorescent rosamine compound selectively stains pluripotent stem cells. Angew. Chem. Int. Ed. Engl. 49, 7497-500.

Klein, C., Fishel, G., 2004. Neural Stem Cells: Progenitors or Panacea? Dev. Neurosci. 26, 82–92.

Levi, B.P., Yilmaz, O.H., Duester, G., Morrison, S.J., 2009. Aldehyde dehydrogenase 1a1 is dispensable for stem cell function in the mouse hematopoietic and nervous systems. Blood 113, 1670–1680.

Malatesta, P., Appolloni, I., Calzolari, F., 2008. Radial glia and neural stem cells. Cell Tissue Res. 331, 165-178.

Marcato, P., Dean, C.A., Giacomantonio, C.A., Lee, P.W., 2011. Aldehyde dehydrogenase - Its role as a cancer stem cell marker comes down to the specific isoform. Cell Cycle 10, 1378-1384.

Matsumata, M., Sakayori, N., Maekawa, M., Owada, Y., Yoshikawa, T., Osumi, N., 2012. The effects of Fabp7 and Fabp5 on postnatal hippocampal neurogenesis in the mouse. Stem Cells. 30, 1532-1543

Morshead, C.M., van der Kooy. D., 2004. Disguising adult neural stem cells. Curr. Opin. Neurobiol. 14, 125-131.

Obermair, F.J., Fiorelli, R., Schroeter, A., Beyeler, S., Blatti, C., Zoerner, B., Thallmair, M.A., 2010. A novel classification of quiescent and transit amplifying adult neural stem cells by surface and metabolic markers permits a defined simultaneous isolation. Stem Cell Res. 5, 131-143.

Oliver-De la Cruz, J., Ayuso-Sacido, A., 2012. Neural Stem Cells from Mammalian Brain: Isolation Protocols and Maintenance Conditions, Neural Stem Cells and Therapy, Dr. Tao Sun (Ed.)

Panchision, D.M., Chen, H., Pistollato, F., Papini, D., Ni, H., Hawley, T.S., 2007. Optimized Flow Cytometric Analysis of Central Nervous System Tissue Reveals Novel Functional Relationships Among Cells Expressing CD133, CD15, and CD24. Stem Cells 25, 1560–1570.

Pfenninger, C.V., Roschupkina, T., Hertwig, F., Kottwitz, D., Englund, E., Bengzon, J., Jacobsen, S.E., Nuber, U.A., 2007. CD133 is not present on neurogenic astrocytes in the adult subventricular zone, but on embryonic neural stem cells, ependymal cells, and glioblastoma cells. Cancer Res. 67, 5727-5736.

Ruff, C.A., Fehlings, M.G., 2010. Neural stem cells in regenerative medicine: bridging the gap. Panminerva Med. 52, 125-147.

Schmid, R., Yokota, Y., Anton, E.S., 2006. Generation and Characterization of Brain Lipid-Binding Protein Promoter-Based Transgenic Mouse Models for the study of radial glia. Glia 53, 345-351.

Singec, I., Knoth, R., Meyer, R.P., Maciaczyk, J., Volk, B., Nikkah, G., Frotscher, M., Snyder, E. Y., 2006. Defining the actual sensitivity and specificity of the neurosphere assay in stem cell biology. Nat. Methods 3, 801-806.

Storms, R.W., Trujillo, A.P., Springer, J.B., Shah, L., Colvin, O.M., Ludeman, S.M., Smith, C., 1999. Isolation of primitive human hematopoietic progenitors on the basis of aldehyde dehydrogenase activity. Proc. Natl. Acad. Sci. USA 16, 9118-9123.

Sun, Y., Kong, W., Falk, A., Hu, J., Zhou, L., Pollard, P., Smith, A., 2009. CD133 (Prominin) negative human neural stem cells are clonogenic and tripotent. Plos One 4, e5498.

Vukovic, J., Bedin, A.S., Bartlett, P.F., Osborne, G.W., 2013. A novel fluorescent reporter CDy1 enriches for neural stem cells derived from the murine brain. Stem Cells Dev.22, 2341-2345.

Yun, S.W., Leong, C., Zhai, D., Tan, Y.L., Lim, L., Bi, X., Lee, J.J., Kim, H.J., Kang, N.Y., Ng, S.H., Stanton, L.W., Chang, Y.T., 2012. Neural stem cell specific fluorescent chemical probe binding to FABP7. Proc. Natl. Acad. Sci. USA 109, 10214-10217.

Neural stem cell isolation from the whole mouse brain using the novel FABP7-binding fluorescent dye, CDr3

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Supplementary information

- Supplementary Materials and Methods
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- Supplementary Figure 5. Gene expression in embryo and adult brain cells sorted by CD133, AldeFluor and CDr3.
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Supplementary Materials and Methods

Confocal microscopy

For whole neurosphere imaging, P1 neurospheres were dissociated and plated in growth media for 2 days in the presence of 500 nM of CDr3. For embryo derived brain cell imaging, whole brains from E14 embryos were triturated to a single cell suspension and stained for 1 hour in 500 nM of CDr3. All confocal imaging was carried out using the Zeiss LSM 510 Meta at 60X magnification.

FACs sorting of cell populations

Cells derived from the E14 embryo brain were labeled with CDr3, AldeFluor, CD133 or SSEA-1. Populations of single or double positive cells that were clearly distinguishable within each marker range were sorted out for neurosphere assay and plated at a density of 5000 cells per 12 well plate. The number of spheres formed was counted after 4-6 days.

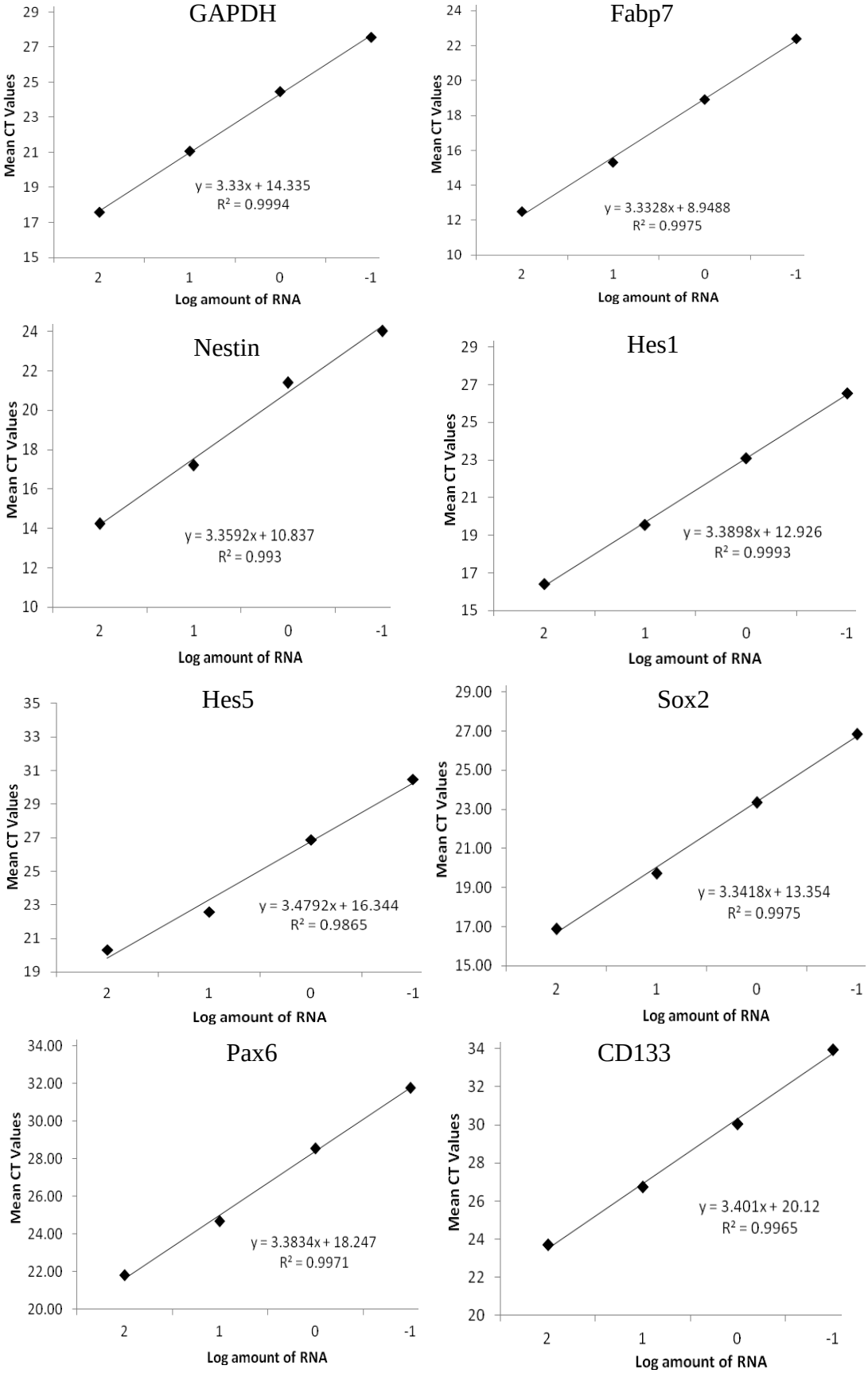
Neuronal differentiation and tripotency testing

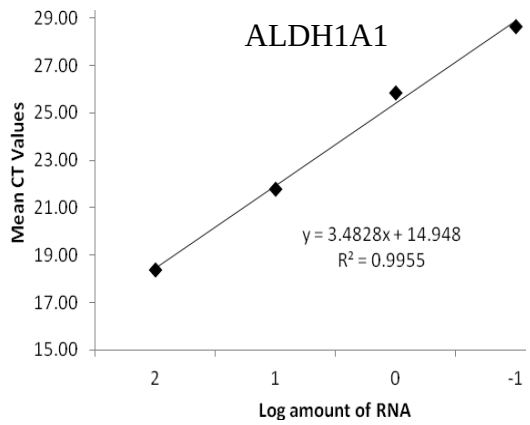
To determine if CDr3 had any detrimental effects on the capacity of neural stem cells to differentiate into neurons, P1 neurospheres were dissociated into a single cell suspension and induced to differentiate in DMEM/F12 + 10% FBS media supplemented with 1X B27 and 50 ng/ml of BDNF (GIBCO) on poly-D-lysine coated plates in DMSO or 500nM of CDr3. After 5-7 days of differentiation, the cells were fixed with 4% paraformaldehyde and immunostained for β-III-tubulin (Sigma) and GFAP (Dako). Images were acquired on the Nikon Ti microscope at 100X magnification. The total number of astrocytes and neurons were counted per region. The average yield of neurons were calculated from a total of 10 regions with an average of approximately 50 cells per region.

qRT-PCR primers

Name	Sequence (5'…3')	Primer efficiency (%)
mGAPDH-F1	aagggctcatgaccacagtc	99.7
mGAPDH-R1	ggatgcagggatgatgttct	
mFabp7-F1	ccagctgggagaagagtttg	99.5
mFabp7-R1	tttctttgccatcccacttc	
mNestin-F1	ggaagaagttcccaggcttc	98.5
mNestin-R1	attaggcaaggggaagaga	
mHes1-F1	acaccggacaaaccaaagac	97.2
mHes1-R1	atgccgggagctatctttct	
mHes5-F1	gcagcatagagcagctgaag	93.8
mHes5-R1	aggctttgctgtgtttcagg	
mSox2-F1	gaacgccttcatggtatggt	99.2
mSox2-R1	tctcggtctcggacaaaagt	
mPax6-F1	agtgaatgggcggagttatg	97.5
mPax6-R1	acttggacgggaactgacac	
mCD133-F1	tgcgatagcatcagaccaag	96.8
mCD133-R1	tttgacgaggctctccagat	
mALDH1A1-F1	ctctgttccccaggtgttgt	93.7
mALDH1A1-R1	catgcaagggtgcctttatt	

Primer efficiency plots


GAPDH
Mean CT Values
Log amount of RNA
y = 3.33x + 14.335
R² = 0.9994
Fabp7
y = 3.3328x + 8.9488
R² = 0.9975
Nestin
y = 3.3592x + 10.837
R² = 0.993
Hes1
y = 3.3898x + 12.926
R² = 0.9993
Hes5
y = 3.4792x + 16.344
R² = 0.9865
Sox2
y = 3.3418x + 13.354
R² = 0.9975
Pax6
y = 3.3834x + 18.247
R² = 0.9971
CD133
y = 3.401x + 20.12
R² = 0.9965



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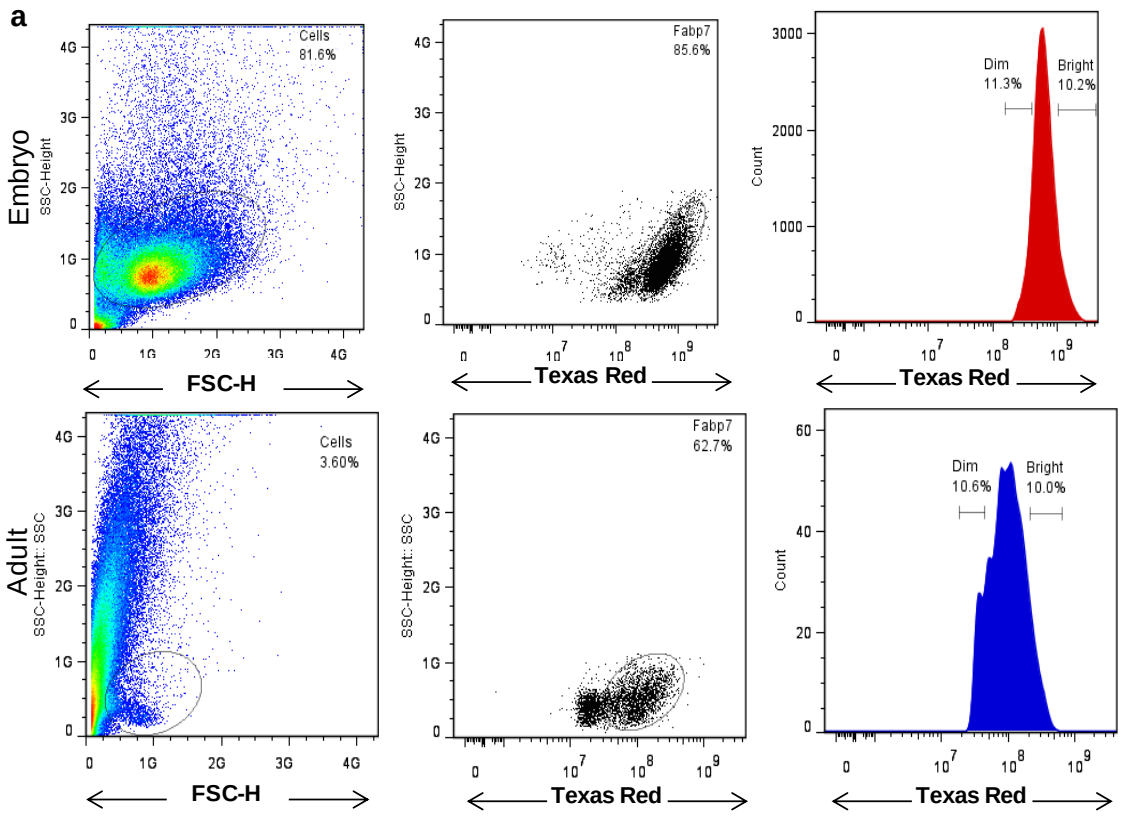
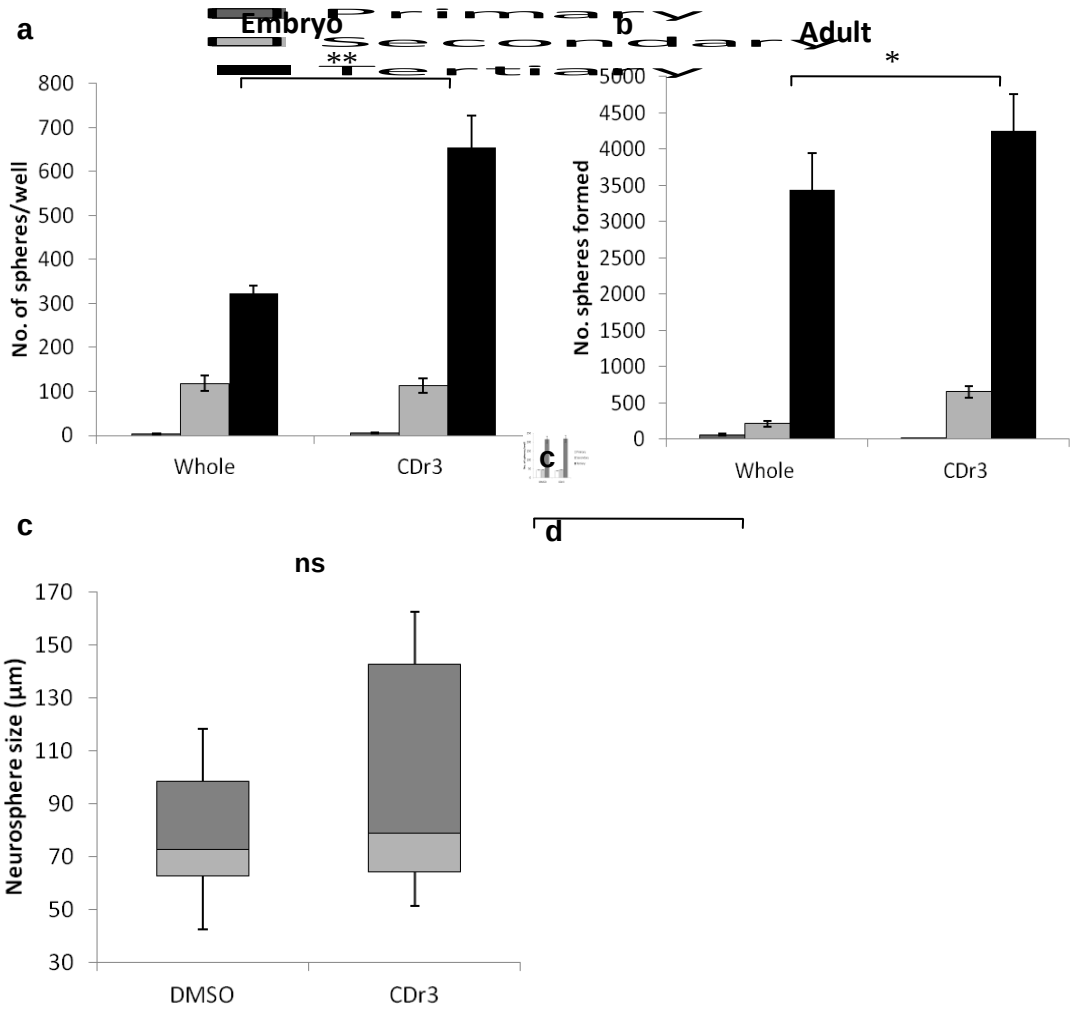


Supplementary Figure 1. Scatter profile of FABP7 and CDr3 stained cells. For both embryo and adult brain derived cells, live cells were pre-gated with size and granularity (Left). A distinct population of CDr3 positive cells were identified by fluorescence (Middle). For sorting, the brightest and dimmest 10% of the cells were gated based on a histogram plot (Right).

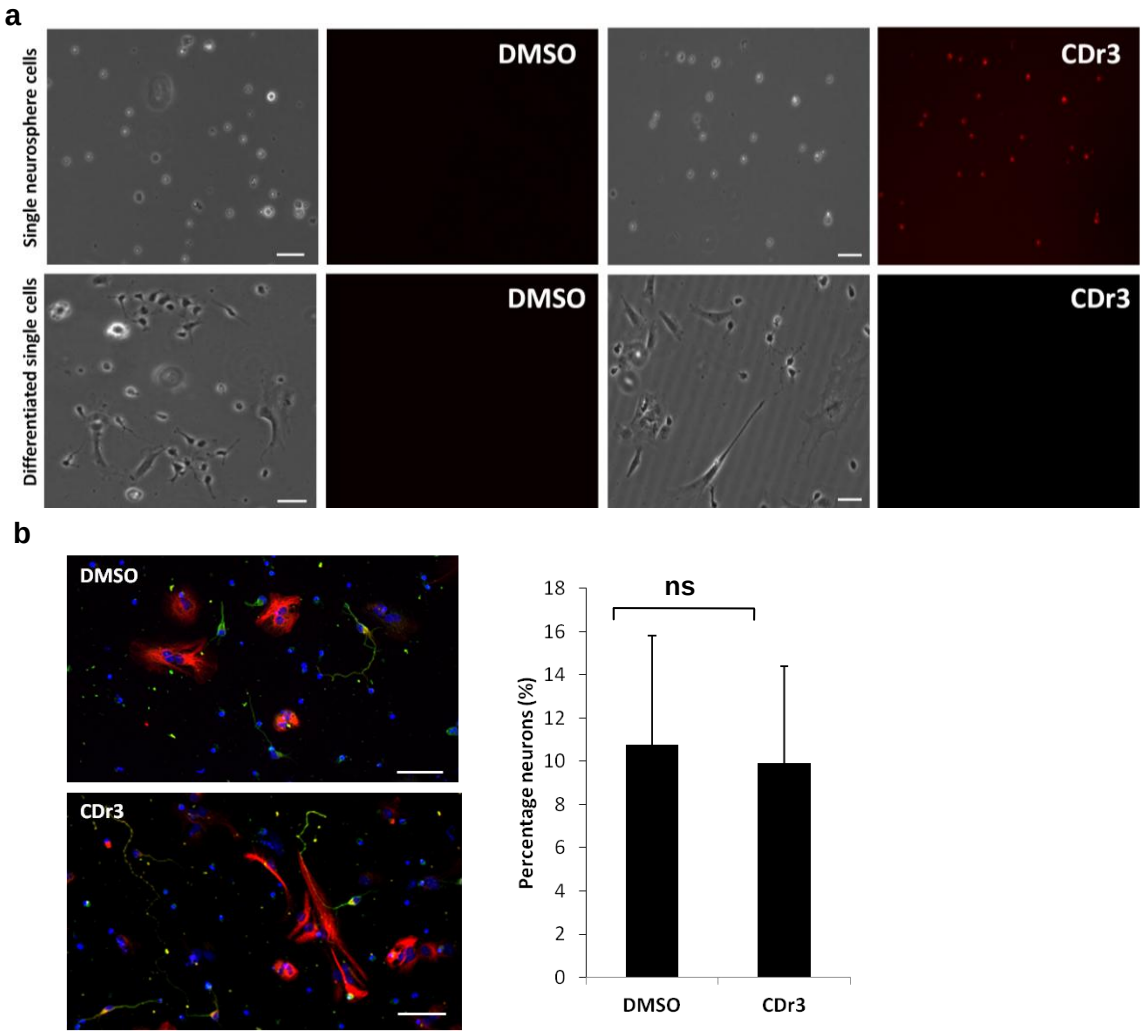
10



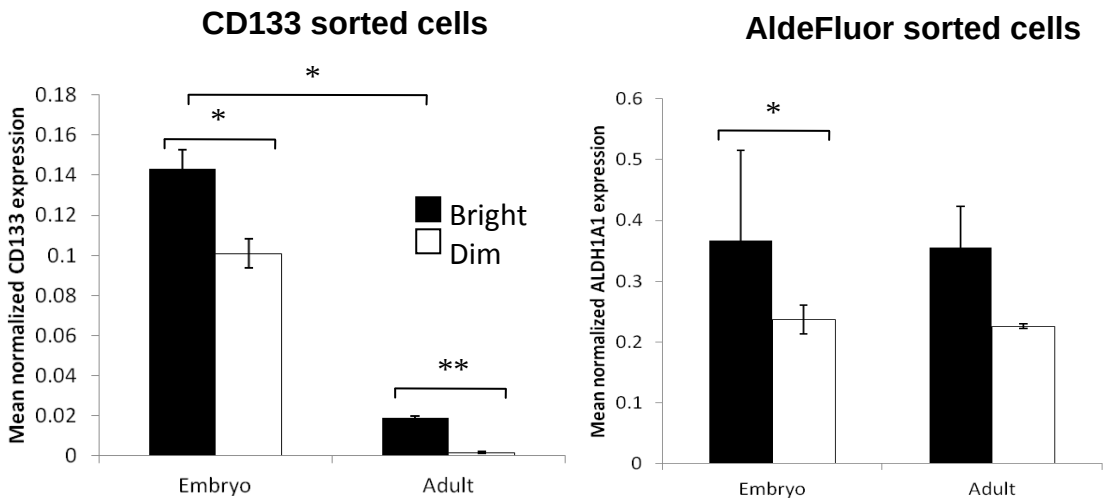
Supplementary Figure 2. Neurosphere growth and size. Neurospheres generated from embryo (a) and adult brain (b) was serially passaged and the number of spheres counted at each passage. CDr3 treated spheres were comparable in sphere size (c) and number (d) with vehicle (0.1% DMSO) treated spheres. An unpaired two tailed t test was performed (* $p<0.05$

and **p<0.01) for mean values. Values shown represent mean ±S.D. For box plot distribution, a Mann-Whitney U test was performed.

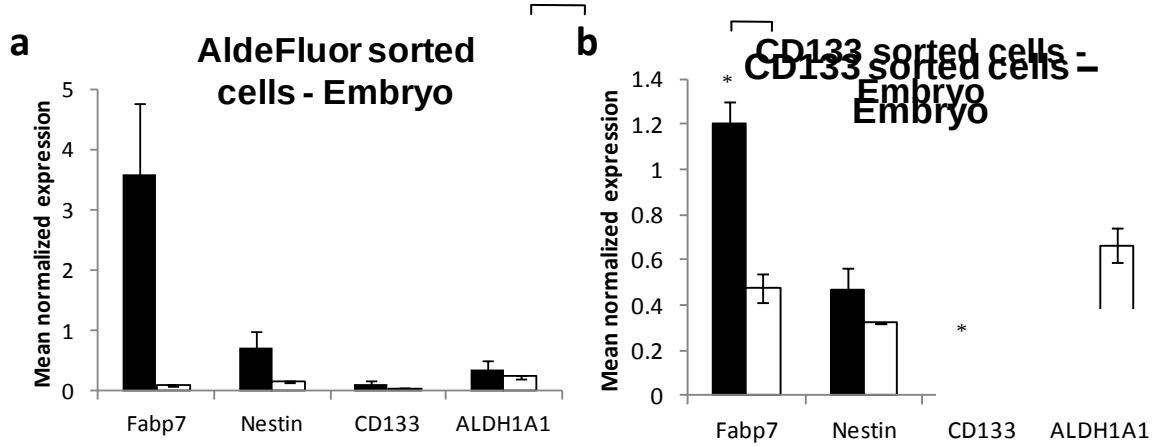
5



Supplementary Figure 3. Neuronal differentiation of neurospheres. (a) Single cells dissociated from neurospheres show bright CDr3 staining but the staining is lost upon differentiation. Scale bar, 50 µm. (b) shows representative images of neurons (green) and astrocytes (red) differentiated from single cells dissociated from neurospheres treated with DMSO and CDr3. Scale bar, 50 µm. The bar graph shows the percentage yield of neurons from DMSO and CDr3 treated dissociated spheres. An unpaired two tailed t test performed shows no difference in neuron yield between the two samples. Values shown represent mean ±S.D.



Supplementary Figure 4. CD133 and ALDH1A1 gene expression in cells from the embryo and adult brain. CD133 expression is robust in the embryo brain but less so in the adult brain. ALDH1A1 expression levels were similar in both the embryo and adult brains for AldeFluor sorted cells. An unpaired two tailed t test was performed (* $p<0.05$ and **$p<0.01$). Values shown represent mean ±S.D.



Supplementary Figure 5. Gene expression in embryo and adult brain cells sorted by CD133, AldeFluor and CDr3. AldeFluor (a) and CD133 (b) sorted cells from the embryo brain were compared for Fabp7, nestin, CD133 and ALDH1A1 expression. An unpaired two tailed t test was performed for bright and dim sorted cells (* $p<0.05$). Values shown represent mean ±S.D.

Supplementary Table 1. Percentage of single marker sorted cells expressing other NSC markers

E14 embryo

%	CDr3	AldeFluor	CD133	SSEA-1
CDr3	100	100	70	40
AldeFluor	10	100	5	5
CD133	100	100	100	20
SSEA-1	25	10	15	100

Adult

%	CDr3	AldeFluor	CD133	SSEA-1
CDr3	100	100	100	100
AldeFluor	10	100	50	60
CD133	30	100	100	10
SSEA-1	40	40	70	100

The respective marker expression in embryo and adult brain cell populations was characterized by flow cytometry. The shaded row in the tables indicates the markers by which the cells were sorted and the unshaded column indicates the markers for which the sorted cells were characterized by immunocytochemistry followed by flow cytometry.

Supplementary Table 2. Subpopulation neurosphere yields from single and double NSC marker sorted cells.

Subpopulation markers	No. of spheres
SSEA-1 +/AF-	150
SSEA-1 +/CDr3^{Bright}	94
SSEA-1+/CD133 -	37
CD133+/SSEA-1 -	45
CD133+ /CDr3^{Bright}	114
AF+ /CDr3^{Bright}	52
AF+/CD133-	24

5 Cells derived from the E14 embryo brain were co-labelled with CDr3, AldeFluor, CD133 or SSEA-1. Table 2 shows the populations of single of double positive cells that were clearly distinguishable within each marker range that was sorted out for neurosphere assay.