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The Adult Mouse Dentate Gyrus Contains Populations of Committed Progenitor Cells that are Distinct from Subependymal Zone Neural Stem Cells

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Key Words. Dentate gyrus • Subependymal zone • Neural stem cell • Self-renewal • Multipotentiality

ABSTRACT

There is currently a debate as to whether or not a neural stem cell (NSC) exists in the adult mammalian hippocampus. Clonal colony-forming assays allow single cells to cells to be evaluated for stem cell properties: self-renewal and multipotentiality. In these in vitro assays, single cells from the subependymal zone (SEZ) of the adult lateral ventricle yield large colonies which self-renew and are multipotential, while single cells from the adult dentate gyrus (DG) produce small, unipotent, and nonself-renewing colonies. We find that multipotential and long-term self-renewing colonies can be isolated only from the early embryonic hippocampus, before the formation of the DG. No movement of progenitors from the postnatal SEZ to the newly forming DG subgranular zone is detected and adult DG colonies in vitro originate from the embryonic hippocampal primordium. These data support a model where embryonic hippocampal NSCs change their properties as the organism ages. When adult DG spheres are cocultured with embryonic brain slices, self-renewal (but not multipotentiality) is restored and maintained for several passages off of slices. Adult clonal DG spheres grown on embryonic brain slices or transplanted into brains of neonatal mice do not give rise to neurons. Neurons arise from separate, small clones that are approximately 10 times more frequent than sphere colonies in vitro and may be responsible for maintaining neurogenesis in the adult in vivo. We propose that there are separate glial and neuronal clones in the adult hippocampus, with glial progenitors being the most proliferative in culture. STEM CELLS *2011;29:1448–1458*

Disclosure of potential conflicts of interest is found at the end of this article.

INTRODUCTION

The sites of ongoing neurogenesis in the adult rodent are the olfactory bulb (OB) and the dentate gyrus (DG) of the hippocampus. Lateral ventricle (LV) neural stem cells (NSCs) and DG precursors are glial fibrillary acidic protein (GFAP)/Nestin/Sox2-positive radial glia-like progenitors [1–3]. NSCs in the subependymal zone (SEZ) of the LV produce precursors that move along the rostral migratory stream and differentiate into OB inhibitory interneurons [1]. In the DG, new neurons originate from precursors in the subgranular zone (SGZ) and progenitors migrate a short distance into the overlying granule cell layer and mature into excitatory neurons [2, 4]. Neurogenesis in both regions is modulated by learning, behavior, and environment [5–8], often with differential responses between the DG and LV [9, 10].

Differences in precursor cell properties also are apparent in culture, where single cells are removed from influences of their respective niches. SEZ NSCs can be readily cultured and clonally expanded indefinitely as multipotential neurospheres [11, 12]. Isolation of DG clones has provoked debate as to whether these precursors possess the cardinal properties of multipotentiality and self-renewal and thus whether they can be termed stem cells. In particular, consistent results have not been achieved [13–16] due to a lack of standardization in dissection technique for cell isolation and also by a lack of clonal assays.

In this study, the properties of DG in vitro colony-forming cells were investigated using precise dissections and single cell-based cultures. As DG colony-forming cells are studied from the embryo to the adult, they gradually lose multipotency and the ability to self-renew, until only glial- or neuronal-restricted clones are present in the adult. Self-renewal, but not multipotency, can be restored by coculture with embryonic brain slices. We conclude that DG precursors are not stem cells, and are distinct from SEZ NSCs. This work highlights the importance of deriving a pure population to study biology of neural precursors in vitro. Studying the properties of DG progenitors in culture is important both for understanding basic mechanisms of proliferation and differentiation and also for developing techniques to expand and maintain cells in culture for use in drug screening or transplantation.

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Materials and Methods

DG Dissection

Adult DG cultures were derived from 2 to 3 months old female CD1 mice (Charles River, Wilmington, MA, www.criver.com) unless otherwise stated. Embryonic dissections were performed from timed pregnant CD1 mice (Charles River). Experimental procedures were performed in accordance with the Guide to the Care and Use of Experimental Animals and approved by the Animal Care Committee at the University of Toronto.

Dissections were performed as described previously [13]. Briefly, brains were embedded in 2% low gelling temperature agarose (Sigma-Aldrich, St Louis, MO, www.sigmaaldrich.com) for 30 minutes at 4°C, then sectioned on a Vibratome at 400–500 μm. LV SEZ and DG were removed under a dissecting microscope (Zeiss, Jena, Germany, www.zeiss.com). The term SEZ is used to avoid confusion with the embryonic subventricular zone (SVZ), though SEZ and SVZ are both used to describe the location of adult LV NSCs [17]. Tissue was dissociated using trypsin 13,000 U/10 mL, hyaluronidase 1,300 U/10 mL, and kynurenic acid 1.33 mg/10 mL for 25 minutes at 37°C then triturating using a glass pipette. Cells were assessed for viability using Trypan blue exclusion immediately before plating at 10 cells per microliter in serum-free media [18] with 10 ng/mL fibroblast growth factor (FGF2), 20 ng/mL epidermal growth factor (EGF) and 2 μg/mL heparin (Sigma) and B27 (Invitrogen, Carlsbad, CA, www.invitrogen.com) in 24-well plates (Nunclon, Rochester, NY, www.nuncbrand.com). Embryonic/early postnatal brains were sectioned at 300 μM and a single cell suspension was obtained without enzymatic treatment. Cultures were not refed or otherwise disturbed until colonies were counted at 7 days. Targeted and nontargeted control short interfering RNA was used at 0.5 μM with Dharmafect transfection reagent 1 (Dharmacon, Lafayette CO, www.dharmacon.com). Knockdown was confirmed by quantitative polymerase chain reaction (qPCR) for *Hes5* and levels were reduced to approximately 60% of control at 48 hours after transfection.

Neurosphere Differentiation

Single neurospheres were plated in individual wells of 48-well plates coated with Matrigel (1/24, BD Biosciences, Franklin Lakes, NJ, www.bdbiosciences.com) in serum-free media containing 1% fetal calf serum (Invitrogen) and B27 and processed for immunocytochemistry after 7 days. Astrocytes for coculture were obtained from postnatal day 1/2 (P1/2) dsRed mice (Jackson, Stock#005441, Bar Harbor, ME, http://jaxmice.jax.org) as described previously [19] and passaged onto 12 mm^2 polycarbonate cell culture inserts (Millipore, Billerica, MA, www.millipore.com) or 24-well plates (Nunclon). When noted, primary spheres were grown and differentiated in the presence of 100 ng/mL brain-derived neurotrophic factor (BDNF, Sigma). BDNF bioactivity was verified in primary cultures of DG granule cells from P5 mice [20]. Briefly, the granule cell layer was dissected from brain slices and cultured in Neurobasal media (Invitrogen) without B27 or without 100 ng/mL BDNF at 10,000 cells per well in 96-well plates.

Neurosphere Passaging

Single clonally derived neurospheres were dissociated using manual trituration, then replated in serum-free media containing B27, FGF2, EGF, and heparin in individual wells of 24-well plates. Dissociation into single cells was verified by inspection, and incompletely dissociated colonies were not included.

Histone2B-GFP Label-Retention

Histone2B-green fluorescent protein (GFP)/M2 mice (a gift from Dr. Konrad Hochedlinger) [21] aged 2–3 months were anesthetized using isoflurane and implanted with 7-day, 0.5 μL micro-osmotic pumps (Alzet, Cupertino, CA, www.alzet.com) containing 4 mg/mL doxycycline in 0.9% saline or saline alone. Pumps were connected to unilateral cannulas at 0.9 mm lateral and 0.2 mm posterior relative to bregma to infuse intracerebroventricularly and were removed after 4 days. DG cells pooled from three to six animals were analyzed for each time point.

Adenoviral Labeling of SEZ Cells

P3/4 RosaYFP (Jackson, Stock#006148) pups were anesthetized with isoflurane and 0.3–0.4 μL adenovirus-expressing Cre recombinase (1×10^{11} pfu/mL, Vector Biolabs, Philadelphia, PA, www.vectorbiolabs.com) was injected unilaterally using a 0.5-μL Hamilton syringe (Reno, NV, www.hamiltoncompany.com) approximately 0.5 lateral and 0.1 posterior to bregma to target the anterior LV.

In Utero Electroporation

Electroporation was performed as described previously [22]. Pregnant CD1 mice (embryonic day (E)14/15) were anesthetized with isoflurane and embryos were injected unilaterally into the LV using a glass pipette through the uterine wall with 2 μL of a solution containing PiggyBAC GFP plasmid with transposase to allow integration [23] along with 0.05% Trypan blue. Five pulses of 40 mV delivered using a square electroporator CUY21 EDIT (Nepagene, Ichikawa, Japan, www.nepagene.jp/E/Eindex.htm).

Slice Coculture

Approximately 300 μM slices of adult or E14 brain were cultured on Transwell collagen-coated 3 μm pore cell culture inserts (Corning, Lowell, MA, www.corning.com/lifesciences) in Neurobasal-A with B27, 0.5 mM L-glutamine, and penicillin/streptomycin (Invitrogen). Single spheres from adult enhanced yellow fluorescent protein (EYFP) mice (Jackson, Stock#005483) were placed onto slices using a dissecting microscope. Media was changed once during the 6–7-day-culture period. Media from untreated slices was used in conditioned media (CM) experiments at one-third the total volume. To obtain secondary spheres, slices were dissociated using only trituration and plated as a single cell suspension in serum-free media with B27, EGF, FGF2, and heparin.

Sphere Cell Transplantation

P1/2 CD1 pups were anesthetized using isoflurane and injected with 10,000 dissociated primary adult DG cells from EYFP mice in 1.0 μL saline using a 5.0-μL Hamilton syringe. Injections were made unilaterally, approximately 1.5 mm lateral to lambda, and 1.5 mm below the dura to target the hippocampus.

Immunocytochemistry/immunohistochemistry

Cultured cells were fixed with 4% paraformaldehyde for 20 minutes at room temperature. For staining of frozen sectioned brains, animals were perfused with phosphate-buffered saline (PBS) then 4% paraformaldehyde. Brains were postfixed in 4% paraformaldehyde overnight at 4°C, then transferred into 30% sucrose for a further day before being embedded in Tissue freezing medium (Triangle Biomedical, Durham, NC, www.trianglebiomedical.com). Antibodies used were Ms anti-Tuj1 (1:500, Sigma), Ms anti-GFAP (1:400, Sigma), Rb anti-GFAP (1:1000, Dako, Glostrup, Denmark, www.dako.com), Rb anti-GFP (1:500, Invitrogen), Ms anti-O4 (1:150, Millipore), Ms anti-Nestin (1:400, Millipore), Ms anti-NeuN (1:100, Millipore), and Ms anti-Map2 (1:200). For NeuN, slides were pretreated by boiling in 10 mM sodium citrate buffer, pH 6.0 for 10 minutes. Goat secondary antibodies (1:400, Alexa, Invitrogen) were used for detection. Nuclei were counterstained with Hoechst (Sigma). Cells were visualized using a Zeiss Axiovert inverted fluorescence microscope and images were acquired with AxioVision v4.6 imaging software and AxioCam MRm camera with monochrome charge-coupled device (CCD) sensor. Confocal images were acquired with an Olympus Fluoview (Center Valley, PA, www.olympus.com) 1,000 and FV10-ASW 2.0 imaging software.

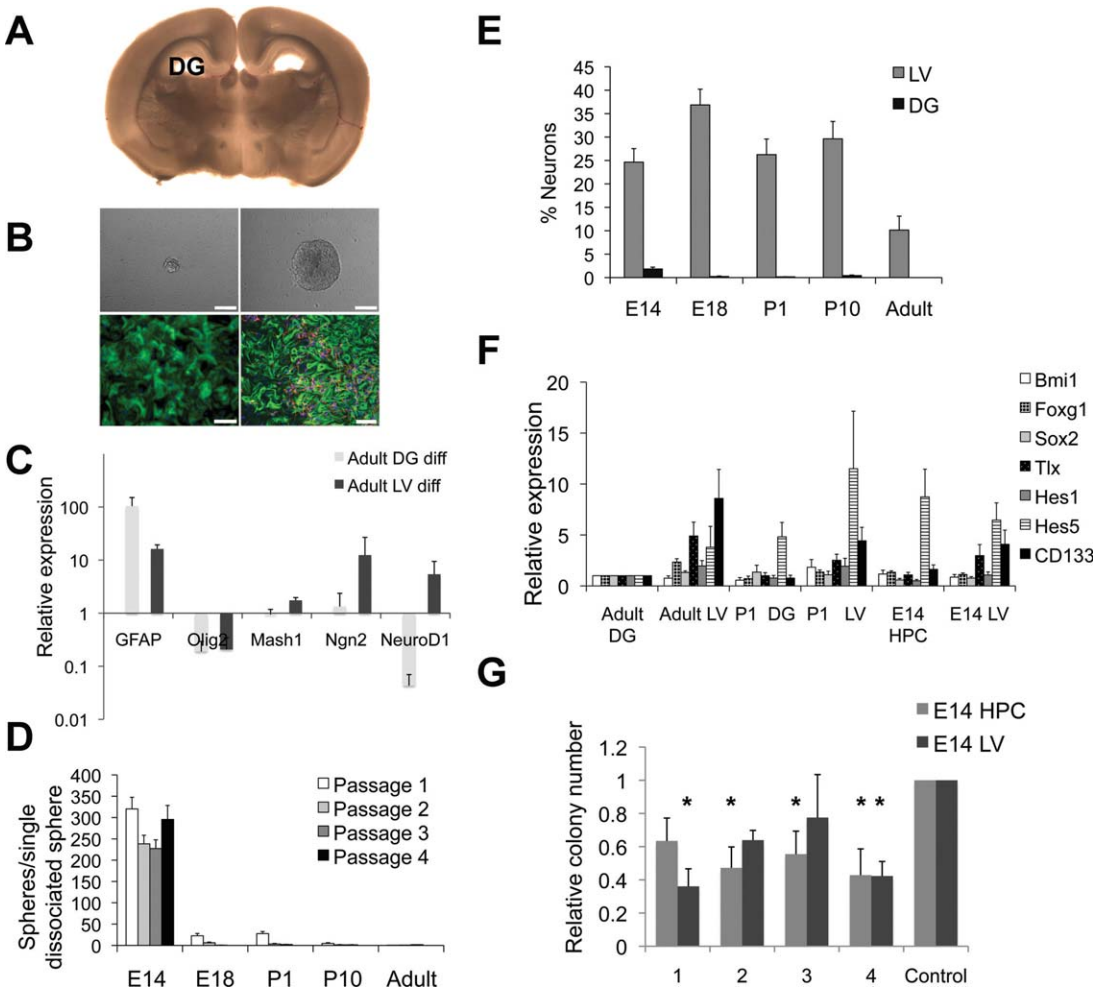


Figure 1. Dentate gyrus (DG) and subependymal zone (SEZ) colonies are distinct. **(A):** Dissection of DG tissue from thin slices avoids SEZ contamination. **(B):** Typical relative size (upper panels) and differentiation potential (lower panels) of adult DG (left) and SEZ spheres (right). DG spheres differentiate into GFAP-positive astrocytes (green), while SEZ spheres produce astrocytes and Tuj1-positive neurons (red). Nuclei are stained with Hoechst (blue). Scale bar = 100 μm. **(C):** Differentiated spheres from the adult DG did not upregulate mRNA for genes important for neuronal differentiation, in contrast those from the adult SEZ. Expression is relative to starting DG or SEZ spheres. **(D):** Clonal spheres from the DG show limited ability to self-renew even in young animals. Sustained passaging of single colonies was only observed in cultures from the early hippocampal primordium at E14. **(E):** DG or hippocampal primordium colonies are multipotential at early stages but not in adults and give rise to fewer neurons at all ages than lateral ventricle (LV) SEZ colonies. **(F):** Adult DG colonies expressed lower levels of mRNA for genes implicated in neurogenesis and self-renewal than those from adult SEZ and were the only sample with low *Hes5* expression. **(G):** siRNA knockdown of *Hes5* function inhibited colony formation from E14 hippocampus and LV, **(1–4)** are *Hes5* specific siRNAs. * indicates significant reduction relative to control, nontargeting siRNA. Data are shown as mean + SEM. Abbreviations: DG, dentate gyrus; E, embryonic day; GFAP, glial fibrillary acidic protein; HPC, hippocampus; LV, lateral ventricle; P, postnatal day; P1/10, postnatal day 1/10.

Flow Cytometry

Primary SEZ and DG cells from Nestin-GFP mice (a gift from Dr Grigori Enikolopov) [24] and Histone2B-GFP/rtTA-M2 were sorted for GFP expression using a FacsAria (BD Biosciences). Nontransgenic littermates (Nestin-GFP) or saline treated transgenic mice (Histone2B-GFP/rtTA-M2) were used as negative controls. Cells were counterstained with propidium iodide (2.5 μg/μL, BD Biosciences) to assess viability. Analysis was performed using BD FacsDiva Software.

Quantitative RT-PCR

RNA was extracted using a Qiagen (Hilden, Germany, www.qiagen.com) RNeasy extraction kit with DNase to remove genomic DNA contamination, quantified and reverse transcribed using SuperscriptIII (Invitrogen). PCR was carried out using Taqman Gene Expression Assays (Applied Biosystems, Carlsbad, CA, www.appliedbiosystems.com) in a 7900HT Fast Real-Time PCR System (Applied Biosystems). Quantification was performed using the delta C_t method [25] with rplp0 ribosomal protein as an endogenous control template.

Statistics

Statistical analysis was performed using SigmaStat 3.1. Student's t tests, Mann-Whitney, χ^2 tests, and analysis of variance (ANOVA) were used as appropriate with an overall significance level of 0.05.

Results

DG and SEZ Clonal Spheres are Distinct at All Stages in Development

DG tissue was obtained by microdissecting from 400 to 500 μm brain slices from CD1 mice throughout the rostro-caudal

extent of the hippocampus. No other hippocampal subregions and no SEZ cells were included (Fig. 1A). In agreement with previous results [13], the DG produced infrequent, small, glial-restricted sphere colonies, and the SEZ produced large multipotential sphere colonies (Fig. 1B, 1C). Primary adult DG and SEZ tissue was also obtained from Nestin-GFP mice and subjected to fluorescence-activated cell sorting (FACS) to verify that in both areas, colonies were derived exclusively from Nestin-positive cells (DG frequency 1 in 296 Nestin-positive cells, SEZ frequency 1 in 45 Nestin-positive cells, $n = 3$). Clonal colonies from the adult DG could not be passaged, and when single colonies were differentiated, the majority produced 100% GFAP-positive astrocytes. In rare cases, we found a small number of neurons from a DG colony (six spheres in 133 produced one or a few neurons each, with remaining spheres giving rise to no neurons).

Proliferative, multipotential cells can be isolated from many areas of the embryonic and early postnatal brain [26–28]; however, only NSCs possess both long-term self-renewal ability and multipotentiality. All other cells with limited self-renewal and/or unipotency are termed progenitors or precursors [29]. We asked whether clonal colonies with NSC properties could be isolated from the DG at any stage in development. DG was microdissected from E18, P1, and P10 and the dentate primordium was dissected from E14 CD1 brains. DG colonies from late embryonic/early postnatal brains (E18–P10) demonstrated limited self-renewal, assayed by single sphere passaging (Fig. 1D). A small number of secondary colonies could be generated; however, colonies could not be expanded or maintained at the third passage. The only age at which continuously self-renewing spheres could be isolated from hippocampus was E14. The DG is not yet present, so these colony-forming cells are found within the proliferative dentate neuroepithelium.

Colonies from the LV/SEZ at various ages were consistently multipotential, generating many Tuj1-positive neurons as well as GFAP-positive astrocytes (Fig. 1E). Perinatal and E14 DG colonies often generated neurons; however, the proportion of neural progeny was consistently lower than from LV/SEZ colonies ($F_{1,295} = 509$, $p < .05$). qPCR analysis of sphere colonies revealed differences in genes implicated in self-renewal and neurogenesis between adult DG and SEZ colonies (effect of brain region $F_{1,167} = 17.0$ $p < .05$, Fig. 1F). *Hes5*, *Tlx*, and *Prominin*/*CD133* transcripts were significantly increased ($p < .05$) in LV/SEZ colonies. siRNA-mediated partial knockdown of *Hes5* in highly expressing and self-renewing primary cultures from E14 hippocampus and LV reduced colony formation (Fig. 1G), suggesting a role for Hes5 in self-renewal. The reduction in colony formation to an average of 52.2% ± 6.7% of control levels paralleled the decrease in primary colony number seen during development for untreated DG cells between E14 and the perinatal period (E18 colonies 45.7% ± 7% and P1 41.3% ± 17.8% of E14 colony numbers). Taken together, these results demonstrate the presence of a self-renewing, multipotential NSC in the DG primordium at E14, but only progenitors limited in self-renewal perinatally, and non self-renewing adult DG precursors.

Culture Manipulations Have Minimal Effect on Adult DG Colony Differentiation Potential

Previous studies have suggested that the addition of single factors influences the differentiation of adult DG colonies into neurons. Potassium chloride (KCl) has been shown to increase primary DG sphere numbers and reveal a latent NSC population with neurogenic capacity [16]. We found that KCl significantly increased adult DG colony number both under our media conditions (7.88 ± 3.28-fold increase, $n = 5$) and under the identical conditions used in [16] (4.49 ± 1.06-fold increase, $n = 4$). However, only two of 51 large DG colonies from KCl gave rise to one and nine neurons each. In addition, while a reduction in spheres from LV was reported previously, we found a consistent increase in sphere formation in our media conditions (1.80 ± 0.27-fold, $n = 3$), and in conditions used in [16] (2.05 ± 0.38-fold, $n = 4$). Thus, in KCl a small number of contaminating SEZ cells could give rise to a significant number of spheres, which would be similar in appearance to "activated" DG colonies. This study by Walker et al. used whole hippocampal dissections performed from thick 2 mm brain slices, which has higher potential for SEZ contamination than our DG dissections.

BDNF also has been reported to induce neurogenesis in differentiating adult DG colonies [14]. We found that the addition of 100 ng/mL BDNF to differentiating spheres had no effect on neurogenic ability as seven of 90 colonies gave rise to neurons ($\chi^2_1 = 1.2$ $p > .05$ vs. Six of 102 control colonies). BDNF bioactivity was verified in primary DG granule cell culture, where the number of Map2-positive neurons was doubled at 5 days compared with controls (not shown). Coculture of adult DG spheres with astrocytes also was ineffective in generating sphere-derived neurons (0 neurons from differentiation of 36 colonies).

Rare neurogenic colonies may represent colonies that have fused or been picked up with neuronal clones, particularly if cell culture densities are not low enough to ensure clonal sphere formation [30]. Separate, small neuronal clones have been reported previously [13], and we detected single cells and small clones expressing Tuj1 in primary cultures after 7 days (Fig. 2A). Neuronal clones were more frequent in cultures containing 100 ng/mL BDNF (120% of control, $t_6 = 10$, $p < .05$). Similar to the large sphere colonies ([13] and data not shown), there was no difference in the number of neuronal clones from the most rostral slice of the DG compared with the most caudal (51.4 ± 8.8 of 5,000 cells rostral vs. 51.7 ± 8.1 of 5,000, $n = 5$ animals).

To test whether "multipotential" DG spheres are nonclonal, a mixture of primary cells from nonfluorescent CD1 and EYFP adult mice was cultured at a density of 5 cells per microliter each, and the primary spheres differentiated after 7 days. Most differentiated spheres (79 of 87) contained no neurons. Eight of 87 spheres contained one neuron each, and five of these single neurons were not derived from the donor cell type that composed the remainder of the host sphere (Fig. 2B, 1C). This indicates that the small minority of spheres initially thought to be multipotential are nonclonal mixtures of sphere cells that produce glia and other neuronal-specified cells that are present in primary cultures.

Adult DG Colonies Are Formed from Cells That Are Relatively Quiescent In Vivo

NSCs in the SEZ of the LV are quiescent, estimated to proliferate once every 2 weeks [31]. We asked whether sphere-forming cells in the DG also have a prolonged cell cycle time. Transgenic mice expressing histone2B tagged with GFP under a tetracycline-inducible promoter [21] were used to test whether adult DG spheres are derived from slowly cycling cells. In this model, all cells become GFP-labeled after a 4-day pulse of doxycycline, then dilute the nuclear GFP label as they divide. If spheres form from relatively quiescent cells, they should retain GFP at long time points after the initial labeling.

Doxycycline was infused intracerebroventricularly for 4 days and mice were sacrificed following a chase period of 2

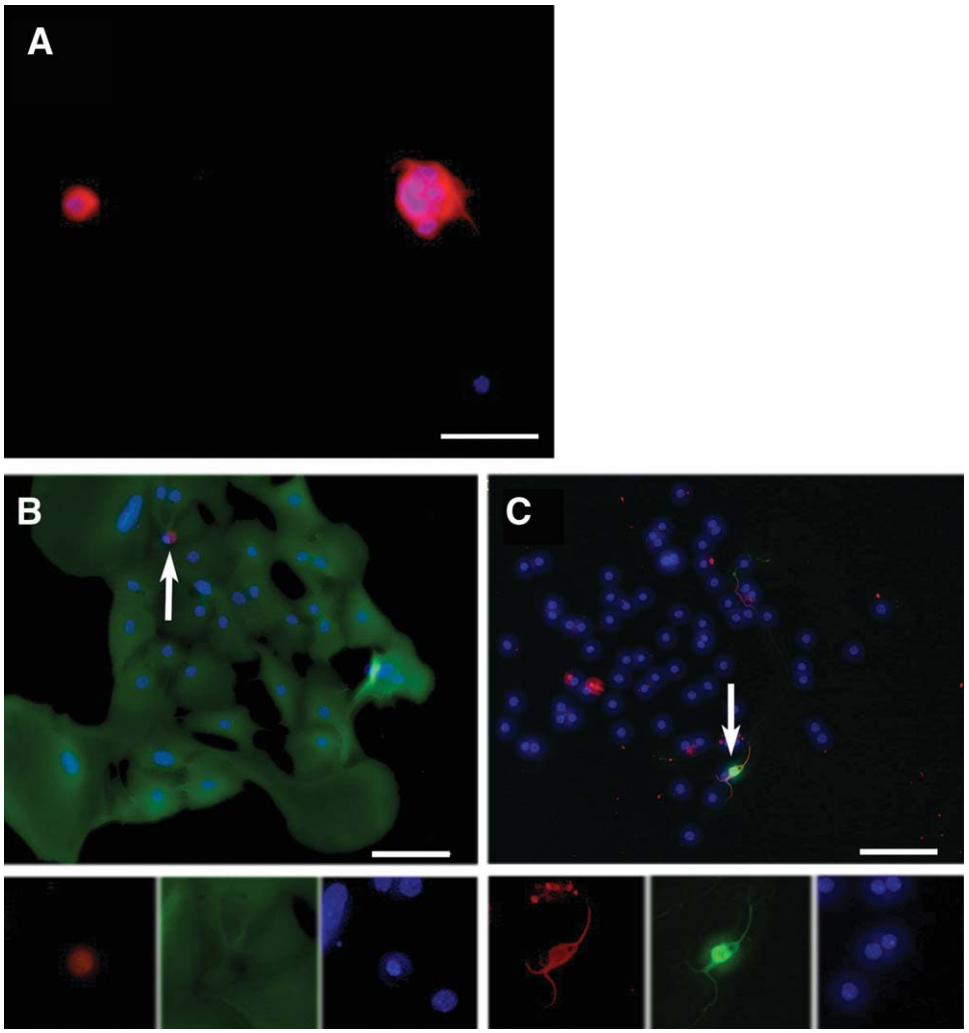


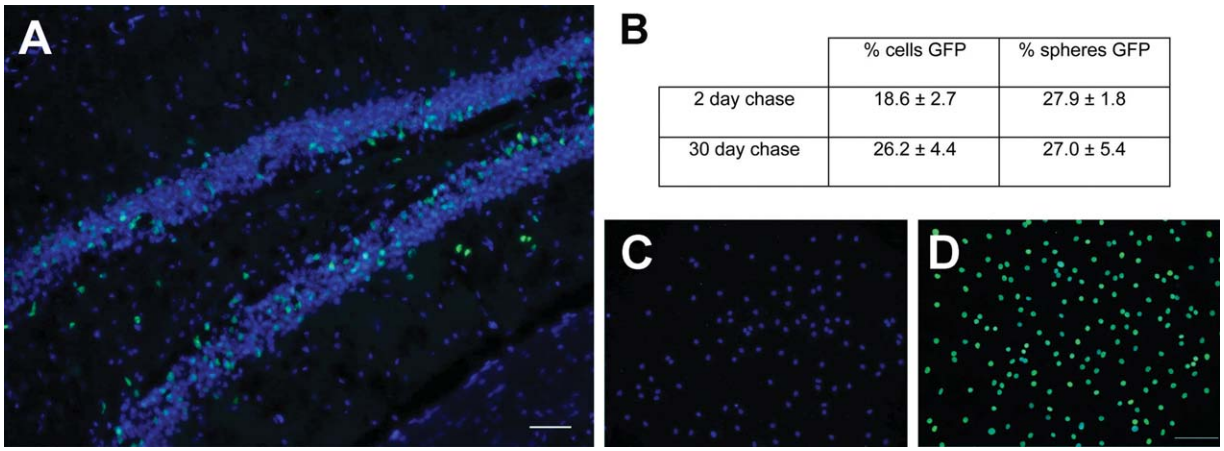
Figure 2. Neuronal clones are found in primary dentate gyrus (DG) cultures. **(A):** Tuj1-positive neurons (red) are present as single cells or small clusters in primary adult DG culture after 7 days in vitro before additional differentiation conditions are applied. EYFP and CD1 (non fluorescent) primary cells plated at an equal ratio resulted in a few spheres containing a neuron that was not derived from the cells that made up the remainder of the sphere **(B)** CD1 neuron in an EYFP-positive sphere, **(C)** EYFP-positive neuron in a CD1 sphere, thus suggesting a small degree of nonclonality of these spheres. Lower panels of **(B)** and **(C)** show cell marked with an arrow-left, Tuj1 (red), middle, EYFP (green), right, Hoechst nuclear stain (blue). Scale bars = 50 μm **(A)**, 100 μm **(B, C)**.

or 30 days. Isolated DG cells were then subjected to FACS (Fig. 3A, 3B). There was a nonsignificant increase in the proportion of GFP-positive cells from 2 to 30 days ($t_4 = 0.44$, $p > .05$), likely reflecting division of transit-amplifying cells to produce labeled progeny that remain in the DG. The proportion of spheres derived from labeled cells was constant between the two time points ($t_4 = 0.89$, $p > .05$), indicating that a proportion of the sphere-forming cells were initially labeled and that none of these diluted that label in 30 days.

GFP is detectable for seven to eight divisions after doxycycline is withdrawn [21]. To rule out the possibility that sphere-forming cells at 30 days after doxycycline were only weakly labeled, spheres were differentiated and stained for GFP (Fig. 3C, 3D). All cells within the sphere were labeled with GFP to varying degrees, indicating that single cells that proliferated several times initially to form a colony were strongly labeled. There also was no shift in GFP fluorescence intensity from 2 to 30 days (not shown). These data show that sphere colonies arise from slowly cycling cells in the DG.

DG Precursors Are Only Derived from the Early DG Primordium

NSCs are found throughout the embryonic nervous system but disappear as the animal reaches mature states [26–28]. We hypothesized that the NSCs detected in the dentate primordium at E14 are lost later in development and that the SGZ is seeded later by separate neural progenitors that underlie adult neurogenesis. The formation of the DG in the embryonic brain begins when a population of cells in the adjacent ventricular lining forms the primary dentate neuroepithelium. These cells then become displaced from the ventricle to form a secondary dentate neuroepithelium. Following this, a unique series of migrations (Fig. 4A) takes place (a) from the secondary dentate neuroepithelium to the granule cell layer, from E15.5 to about the first postnatal week and (b) from a tertiary dentate matrix, also displaced from the SEZ, and below the developing granule cell layer to the SGZ, from E18/19 to about the third postnatal week [32, 33]. A third migration recently has been described, where cells from the area immediately adjacent to the posterior LV move into the DG beginning around P5 [34], suggesting



	% cells GFP	% spheres GFP
2 day chase	18.6 ± 2.7	27.9 ± 1.8
30 day chase	26.2 ± 4.4	27.0 ± 5.4

Figure 3. Dentate gyrus (DG) colonies arise from label-retaining cells. **(A):** Green fluorescent protein (GFP) expression in histone2B/M2 transgenic mice 30 days after induction by doxycycline administration. **(B):** The same proportion of sphere-initiating cells from the adult DG was labeled at 2 or 30 days after doxycycline pumps were removed. **(C):** Differentiated cells from spheres arising from the negative fraction did not express GFP, while progenitor cells in spheres arising from GFP-positive cells contained varying GFP levels, **(D)**. Nuclei are stained with Hoechst. Scale bars = 200 μm **(A)**, 100 μm **(D)**. Abbreviations: GFP, green fluorescent protein.

that a population of posterior SEZ cells migrates in to seed the SGZ with progenitors early in the postnatal period.

To test this hypothesis of SEZ precursors migrating postnatally into the DG, adenovirus-expressing Cre recombinase was injected into the anterior LV of Rosa-YFP reporter mice at P3/4 to permanently label SEZ cells with YFP. By sacrificing some mice 24 hours after injection and culturing cells from the SEZ and DG, we determined that injections had not initially labeled any DG cells but that 8.6% ± 1.5% of anterior SEZ sphere colonies were YFP labeled ($n = 4$). Remaining pups were allowed to survive for an additional 15 days at which time 6.5% ± 1.3% of anterior SEZ colonies remained labeled ($n = 10$). We also verified that posterior SEZ cells adjacent to the DG were labeled by examining sectioned brains (Fig. 4B, 4C) and by deriving cultures from posterior SEZ only (Fig. 4D, 16.0% ± 2.8% labeled, $n = 4$). Despite the high labeling of SEZ spheres, no YFP-positive colonies from the DG dissections were observed (Fig. 4E, $n = 14$), supporting that there is no significant migration of SEZ progenitors to seed the SGZ in the early postnatal DG.

When the unfractionated hippocampus was dissected using an approximately 2 mm thick brain slice from pups injected with the Cre adenovirus, YFP-positive cells were observed in the hippocampal tissue (Fig. 4F, 4G). Subsequently, whole hippocampus cultures gave rise to large and small YFP-positive spheres in both control and KCl conditions (Fig. 4H). Four of four large YFP-positive spheres passaged and were multipotential (Fig. 4I, 4J). Thus, SEZ-derived NSC colonies are present in gross hippocampal cultures.

These results support the hypothesis that sphere-forming progenitors are present in the DG from earlier embryonic stages and have lost ventricular contact before P3. To demonstrate that adult DG progenitors derive from the embryonic DG primordium, in utero electroporation at E14/15 was used to specifically label this region with GFP, as viral injection at this stage would have labeled cells lining the medial and lateral ventricular walls, hitting both LV SEZ and DG progenitors. Cells were electroporated with a GFP PiggyBAC plasmid along with transposase to allow integration. When the DG primordium was dissected 24 hours after electroporation, 13.7% ± 2.4% ($n = 5$) of clonal sphere colonies were GFP labeled. Cultures obtained from mice allowed to survive to 3 weeks of age had an average of 2.6% ± 0.8% ($n = 4$) labeled DG colonies. These data indicate that DG spheres derive from the early embryonic DG primordium, and not later from the adjacent SEZ that contains the LV NSC pool.

Self-Renewal but Not Neurogenesis Can Be Restored by Co-Culture with Embryonic Brain

Multipotential and self-renewing DG progenitors may exist in the adult, but they may be niche-dependent. As single factors were found to be ineffective in inducing multipotentiality and self-renewal in adult DG precursors, we next tested a slice coculture system. This provides exposure to multiple cell types and factors that may be present in the adult DG precursor niche. EYFP-positive adult clonal DG spheres were cultured on nonfluorescent CD1 adult brain slices containing hippocampus for 6–7 days. In this system, we assayed for self-renewal by dissociating slices into single cells and performing a "secondary" clonal EYFP neurosphere assay.

A small number of EYFP-positive secondary adult DG spheres (4.7 ± 0.6, $n = 42$ slices) could be derived from dissociated adult slices; however, these spheres were small, did not give rise to tertiary spheres ($n = 47$ EYFP spheres passaged), and did not produce neurons when differentiated ($n = 25$, one sphere with four neurons was observed). Control, EYFP adult SEZ spheres, or EYFP E14 hippocampal spheres cultured on adult CD1 brain slices gave rise to secondary EYFP spheres that passaged (100%, $n = 35$ adult SEZ spheres and 100% $n = 32$ E14 hippocampal spheres) and gave rise to neurons (86% of passaged adult SEZ spheres produced neurons, $n = 44$ spheres, 100% of E14 hippocampal spheres produced neurons, $n = 22$).

Embryonic forebrain slices may represent a more supportive niche, given that E14 dentate primordium spheres possess both self-renewal and multipotentiality. When adult EYFP DG spheres were cultured in contact with E14 forebrain slices containing hippocampal primordium, we again found a small number of secondary spheres arose clonally directly from dissociated slices (2.5 ± 1.1, $n = 35$ slices). However, when these secondary spheres were dissociated and passaged, 16 of 36 spheres gave rise to more than one tertiary sphere. Single tertiary colonies continued to passage, in some cases producing hundreds of new EYFP-positive colonies (Fig. 5A, 5B) from a single dissociated sphere. However, differentiation of single secondary spheres did not show restored neurogenic capacity (two spheres with one neuron each, $n = 63$ spheres). We find that embryonic slice cocultures restore one NSC

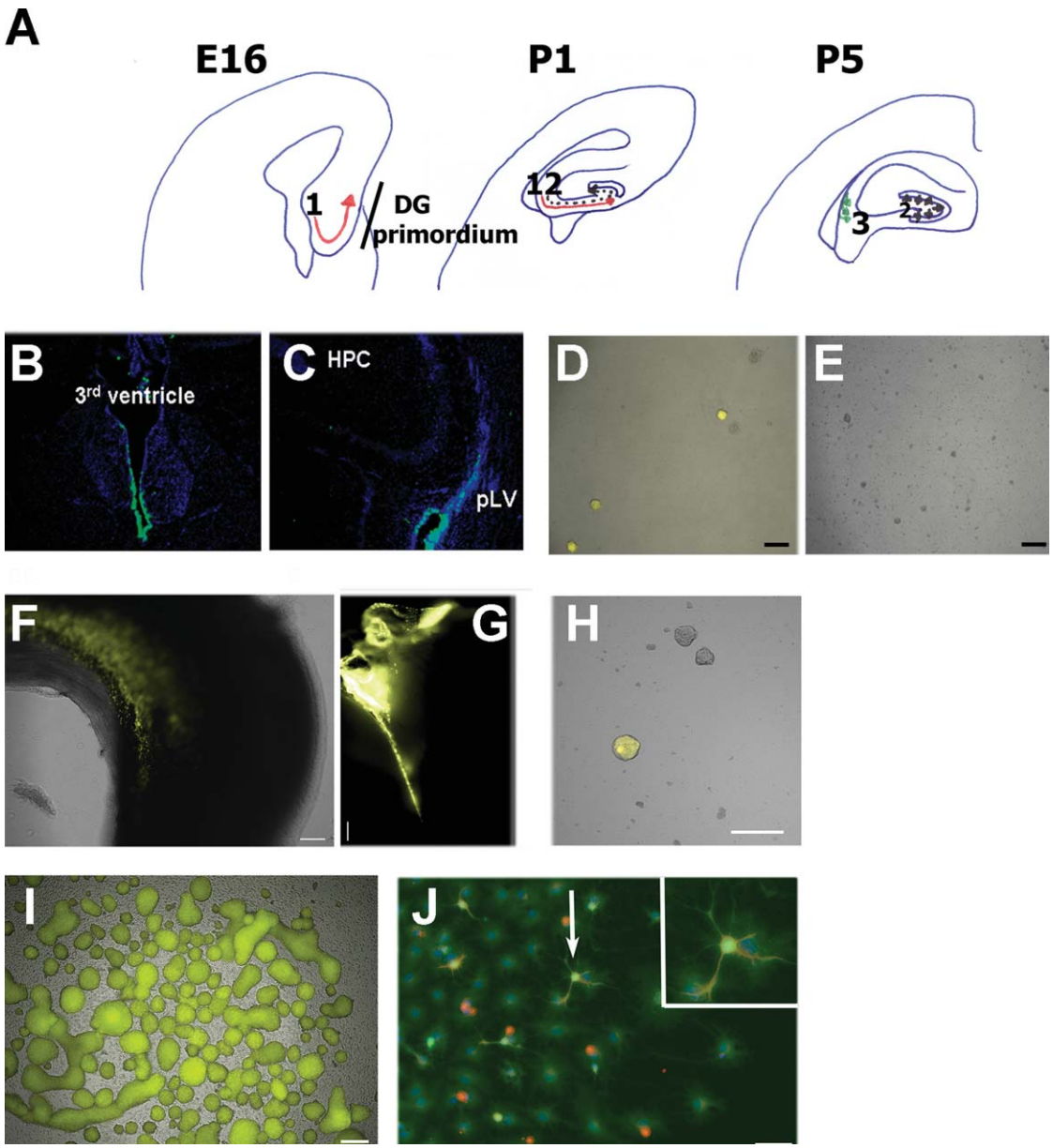


Figure 4. Subependymal zone (SEZ) progenitors do not seed the dentate gyrus (DG) in the early postnatal period. **(A):** Cell migration during DG formation. At E16, the first migration (1, red) is beginning to form the granule cell layer. Later at postnatal day 1 (P1), a second migration (2, dashed black) carries cells beneath the developing granule cell layer. By P5 the first migration is nearly complete and cells from the second migration settle locally to form the subgranular zone. A third migration of cells (3, green) from the hippocampal side of the adjacent posterior lateral ventricle (pLV) has been reported in the early postnatal period. Injection of Ad-Cre into the anterior LV of P3/4 RosaYFP pups gives widespread labeling of the ventricular system **(B)**, third ventricle, **(C)**, pLV. **(D):** YFP-positive spheres were obtained from LV and pLV subependymal zone (SEZ) cultures 2 weeks after injection but not from DG cultures, **(E)**. Gross hippocampal dissections include SEZ-derived neural stem cells. Labeled cells on the surface of a whole dissected hippocampus **(F)** and in the corresponding anterior LV **(G)**. Hippocampal cultures contained YFP-positive colonies **(H)** that were both self-renewing **(I)** and multipotential **(J)**, most YFP cells (green) had astrocytic morphology and a subset stained for Tuj1 (red, arrow). Scale bars = 200 μm **(D–I)**, 100 μm **(J)**. Abbreviations: DG, dentate gyrus; E, embryonic day; HPC, hippocampus; P, postnatal day; P1/5, postnatal day 1/5; pLV, posterior lateral ventricle.

property, self-renewal, while having no influence on multipotentiality. When only CM from slices was used, E14 but not adult CM allowed 7-day-old adult DG spheres to increase in size over a further 7 days in culture (Fig. 5C, $F_{1,175} = 13.5$, $p < .01$). When these 14-day-old spheres were passaged, we obtained a small number of secondary spheres from control, Neurobasal media and a significant increase from E14 CM that was maintained over four passages in the absence of CM (Fig. 5D, $F_{1,186} = 6.11$, $p < .05$). However, the magnitude of this effect was much smaller than in contact coculture, indicating a minor role for soluble factors. qPCR on self-renewing colonies derived from E14 coculture revealed an upregulation of *Hes5* relative to control adult DG spheres (Fig. 5E), suggesting that this factor might be important for restored self-renewal.

Transplantation of Adult DG Progenitors Does Not Restore Neurogenic Ability

As no culture manipulations were found to alter the multipotentiality of adult DG colonies, we next transplanted dissociated EYFP-positive adult DG or SEZ spheres into the

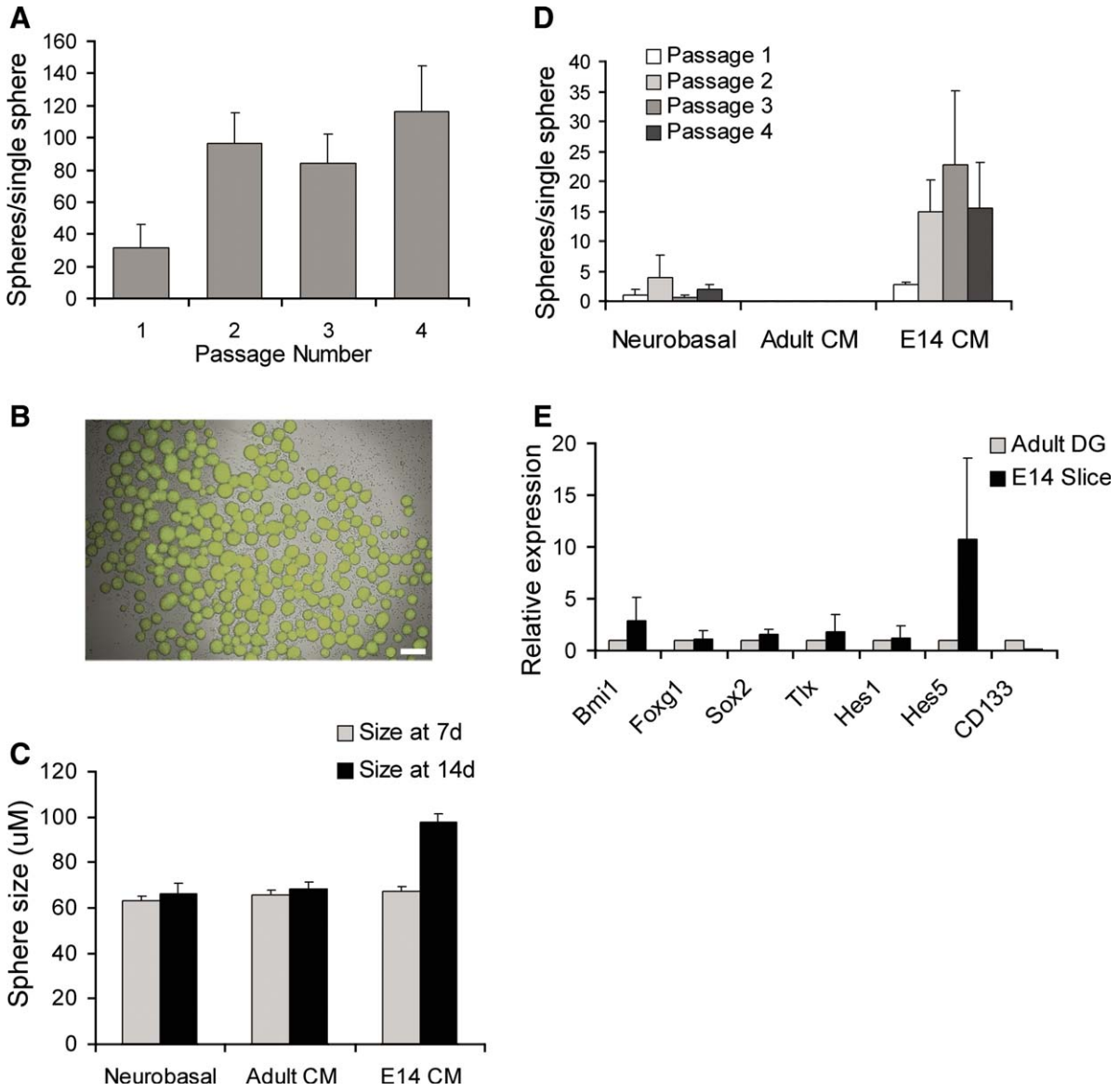


Figure 5. Coculture with E14 forebrain slices restores self-renewal in adult dentate gyrus (DG) spheres. **(A):** Spheres derived from E14 slice cocultures passaged into normal media gave rise to tertiary spheres and continued to passage. **(B):** Single clonal adult DG colonies can give rise to hundreds of secondary spheres after a 6–7-day coculture with embryonic forebrain. Conditioned media from embryonic but not adult slices can increase adult DG sphere size **(C)**, and, to a modest extent, single sphere passaging **(D)**. In these cultures there is an upregulation of *Hes5* in slice-derived spheres (E14 Slice) compared with primary adult DG spheres **(E)**. Data are shown + SEM. Abbreviations: CM, conditioned media, DG, dentate gyrus; E, embryonic day.

hippocampus of P2 CD1 mice. In DG sphere transplants, many EYFP-positive cells survived and the majority expressed glial markers (Fig. 6). No Tuj1-positive cells were observed, and only one single example of an EYFP- and NeuN-positive cell was seen. This observation would be predicted from in vitro differentiation experiments where neurons were occasionally found adhered to spheres. Many SEZ cells transplanted back to the LV migrate and give rise to new neurons [35, 36]. We found that 3.0% of SEZ cells transplanted to the hippocampus expressed NeuN (Fig. 6D, $n = 3$); however, heterotopic transplantation has been previously found to promote glial differentiation [37]. Despite being given the opportunity to form contacts with cellular neighbors and become exposed to soluble factors found in their in vivo niche, DG progenitors were unable to initiate a neurogenic program.

Discussion

There is currently controversy in the NSC field as to whether precursors from the adult rodent DG can be described as stem cells, and how they differ from SEZ NSCs. In this study, clonal assays in vitro were used to test for the stem cell properties of self-renewal and multipotentiality. Using these two criteria, NSCs can be detected in the early embryonic hippocampus but adult DG precursors are lineage-restricted progenitors with limited proliferative capacity.

Long-term self-renewing multipotential colonies were detected in the DG primordium region at E14, but not at any other later time points. These stem cells must either migrate away or differentiate or change their properties. If one of the first two scenarios is true, then a new progenitor cell must

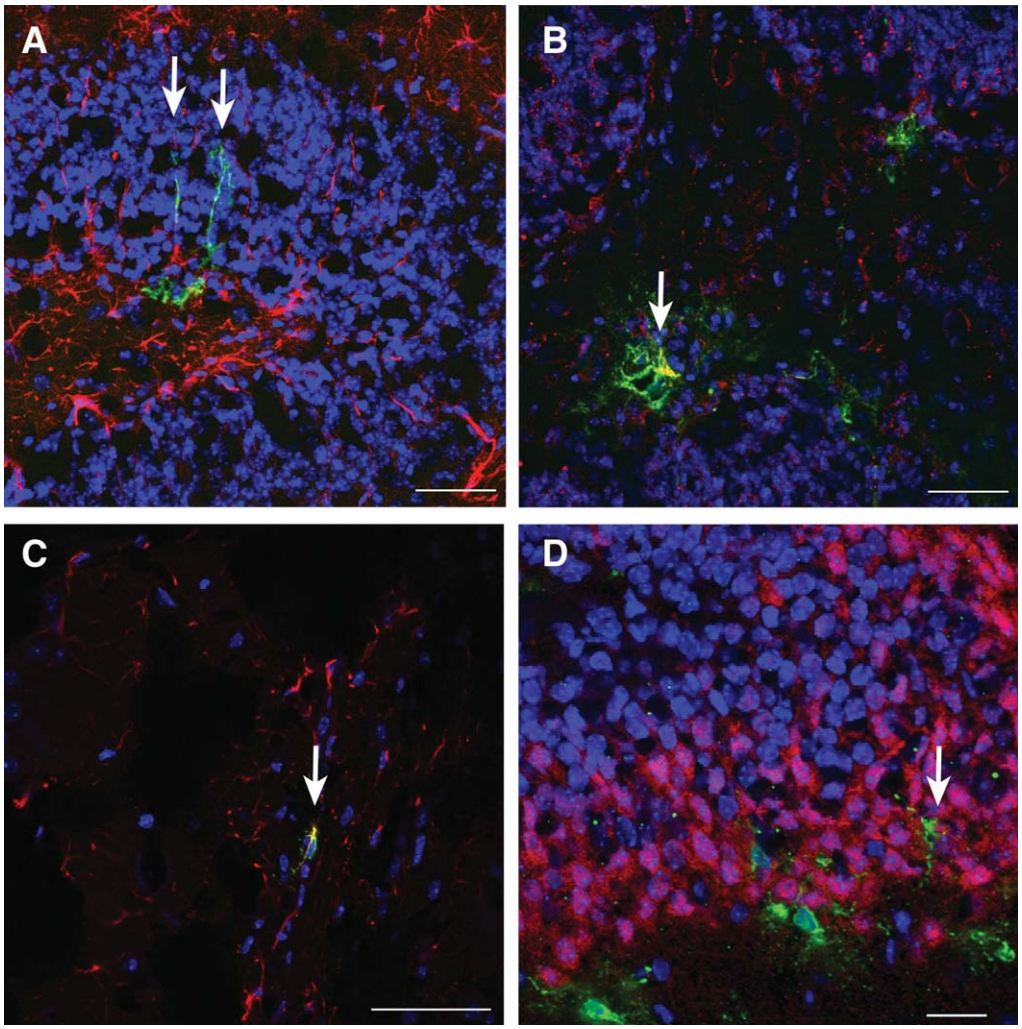


Figure 6. Dissociated primary adult dentate gyrus spheres transplanted into postnatal day 1/2 mice express mainly glial markers. Transplanted YFP-positive cells expressed **(A)** GFAP (astrocytes) 84.2% ± 2.4%, **(B)** O4 (oligodendrocytes) 3.7% ± 1.7%, and **(C)** nestin (progenitors) 6.5% ± 2.3%. **(D):** NeuN-positive cell (neuron) derived from transplanted adult subependymal zone cells. Data are ±SEM. Nuclei are stained with Hoechst. Scale bars = 50 μm **(A–C)**, 20 μm **(D)**.

arrive to seed the DG and support adult neurogenesis. No migration of SEZ progenitors to the DG was detected in the early postnatal period when the SGZ is being formed. However, sphere-forming cells in the adult DG could be labeled by electroporating the hippocampal primordium in the E14/15 brain, suggesting that DG and SEZ lineages are separate early in development, and that there is no significant contribution of SEZ progenitors to the DG after the initial loss of ventricular contact during the formation of the dentate neuroepithelium. The data support a model where DG progenitors resident in the hippocampal primordium gradually lose stem cell properties to become restricted precursors in the adult brain.

The first isolation of DG spheres reported stem cell properties, as colonies from rat hippocampus were observed to self-renew and generate neurons and glia [38]. A subsequent study revealed that mouse or rat hippocampal cultures often contain contaminating SEZ NSCs [13]. The hippocampus is surrounded by regions that give rise to passageable and multipotential colonies, including the third and posterior LV SEZ and hippocampal arch (collapsed embryonic ventricular zone in the dorsal hippocampus). Removing the entire hippocampus (DG and cornu ammon) [14, 15] including the hippocampal arch, makes it difficult to exclude SEZ cells. Contaminating cells at dissection are an important issue, as extensively self-renewing SEZ colonies would eventually dominate cultures. The issue of contamination is especially important in KCl cultures as KCl also increases SEZ sphere number, and the DG "NSCs" supposedly activated by KCl resemble SEZ spheres [16]. Using a precise dissection technique from thin brain slices, primary adult DG spheres arise that have extremely low or no capacity for self-renewal and differentiate only into glia. This study suggests that adult DG precursors do share one characteristic with SEZ stem cells—a prolonged cell cycle time. This correlates well with in vivo observations [2, 39].

If the large clones in culture in fact derive from glial-restricted progenitors and not multipotential stem cells, this implies that there must be separate, neuronal-specific progenitors. Multiple studies from the rodent brain and spinal cord support the existence and in fact predominance of neuron- or glial-restricted progenitors during development [40–47]. Retroviral lineage tracing of Sox2-positive adult DG progenitors in vivo has revealed that the vast majority of clones are solely composed of neurons and only a single clone out of 363 analyzed contained a neuron and an astrocyte [48]. A second

lineage tracing study also identified many clones that contained only neurons or only astrocytes, along with a subset of 10% seemingly multipotential clones [49]. Both studies relied on spatial proximity to define clones, a method which has shown to be unreliable within the rodent brain, as significant migration can occur from a single, initially labeled cell [50]. In addition, it can be difficult to distinguish between mature astrocytes and nonradial, neurogenic DG progenitor cells [48] due to similar marker expression and morphology. This would lead to an overestimation of astrocyte-containing clones. Definitive demonstration of a single cell that gives rise to both neurons and astrocytes will require marking of single clones by a method such as tracking retroviral insertion sites [50] along with careful phenotypic analysis.

An alternative explanation is that in vitro clones grow from multipotential progenitors, but that specific niche factors are required in order to display multipotentiality. Although we cannot completely rule out this possibility, the failure of DG spheres to produce neurons after BDNF, astrocyte coculture, slice coculture, or transplantation suggests that either this is untrue for most clonal adult DG colonies, or that niche factors required to support neurogenesis are difficult to replicate under artificial conditions.

One report of passaged single-cell derived DG colonies giving rise to neurons and astrocytes is evidence that multipotent progenitors exist, although it is unclear how frequently this was observed [51]. Our finding that very few single DG spheres under various differentiation conditions occasionally contain one or a small number of Tuj1-postive neurons hinted that some DG colonies are multipotential; however, it may be explained alternatively. Small Tuj1-positive clones and single neurons are found in primary cultures (without any differentiation media applied), suggesting that they originate from specified neuronal-restricted progenitors or young neurons. We show that neuronal clones can become associated with colonies during primary growth. Further, if BDNF acts specifically on these neuronal-restricted clones [52] to increase their size or frequency, this could artificially increase the appearance of "multipotential" nonclonal spheres [14]. Neuronal clones make up approximately 1% of the total cells plated from primary DG dissections (an estimated 10-fold higher than the numbers of glial-specific clones in vitro) and could account for the large number of new neurons generated in this area in vivo. There are likely a host of factors in addition to BDNF that can promote proliferation or survival of these neuron-restricted clones.

The only culture manipulation to produce lasting changes in adult DG colony properties (inducing self-renewal but not multipotentiality) was coculturing with embryonic forebrain slices. CM and adult brain slices failed to reproduce this effect, indicating that cell contacts with some embryonic cell type(s) are sufficient to induce sustained self-renewal capacity. Interestingly KCl, which mimics electrical stimulation of neural cells, increased DG colony number and size when added to the culture media. The interaction between cells in E14 forebrain slices and adult DG progenitors upregulated expression of the Notch effector Hes5. Several previous studies have implicated Notch activation in NSC properties [53–56] and specifically Hes5 [57, 58]. Neurogenic ability was not increased in the self-renewing spheres, indicating that the two cardinal stem cell properties can be independently regulated. This dissociation of the stem cell properties of self-renewal and multipotentiality reinforces the importance of assaying for both, and using rigorous criteria to define NSCs [29].

Conclusions

Precursor cells within specific adult tissues are rarely studied for regional differences. Here, we show that DG and SEZ progenitor lineages separate early in the developing brain and highlight the distinct properties of adult DG and SEZ clones in culture. We propose that adult DG colony-forming cells currently cannot be classified as NSCs based on our in vitro assays or in vivo lineage tracing assays [48, 49] and that separate progenitors in the adult DG are responsible for neurogenesis and gliogenesis, with gliogenic clones proliferating most in culture. Future in vitro investigations of neural precursors must start with pure populations from either the DG or SEZ to interrogate their unique properties and increase our understanding of the cells that maintain the complex process of adult neural cell replacement. Without this knowledge, attempts to study precursors in vivo or establish cultures for the purpose of transplantation or as disease models will meet with many obstacles.

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Disclosure of Potential Conflicts of Interest

The authors indicate no potential conflicts of interest.

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