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TISSUE-SPECIFIC STEM CELLS

Spontaneous Fusion and Nonclonal Growth of Adult Neural Stem Cells

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Key Words. Adult neural stem cell • Fusion • Neurosphere • Monolayer • Chimera • Nonclonal growth

ABSTRACT

Multipotent neural stem cells (NSCs) can be isolated from various regions of the adult brain and propagated in vitro. Recent reports have suggested spontaneous fusion events among NSCs when grown as free-floating neurospheres that may affect the genetic composition of NSC cultures. We used adult NSCs expressing either red fluorescent protein (RFP) or green fluorescent protein (GFP) to analyze the fusion frequency of rat and mouse NSCs. Fluorescence-activated cell sorting (FACS) revealed that, under proliferating conditions, approximately 0.2% of rat and mouse NSCs coexpressed RFP and GFP irrespective of whether the cells were grown as neurospheres (mouse NSCs) or as attached monolayers (rat and mouse NSCs). Fused cells did not proliferate and could not be propagated, suggesting that aberrantly fused cells are not viable. Furthermore, we found that neither neurospheres nor monolayers grew clonally, because even very low-density cultures had spheres containing both GFP- and RFP-expressing cells and monolayer patches with GFP- and RFP-expressing cells in close proximity. The nonclonal growth between distinct NSC populations strongly suggests the use of careful and precise culture conditions, such as single-cell assays, to characterize potency and growth of NSCs in vitro. STEM CELLS 2007;25:871–874

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

INTRODUCTION

The isolation, expansion, and differentiation of adult NSCs in vitro have allowed the extensive molecular and biochemical characterization of the multipotent progenitors that persist in the adult brain. Furthermore, the in vitro expansion of adult NSCs opened the perspective of a potential therapeutic use of propagated NSCs in human disease. Since the first description of adult NSC culture conditions was published [1], a variety of culturing techniques has been described [2–6]. In principle, NSC can be cultured using two different methods: as neurospheres (which is the most commonly used culture technique) or as monolayers attached to the culture dish surface. We have recently characterized NSCs grown as neurospheres or monolayers and found differences in the growth conditions and cellular properties depending on the respective culture condition [4]. Despite these apparent differences in NSC properties, both culturing methods are commonly used to characterize proliferative capacity, self-renewal, and differentiation potential of NSCs. However, previous reports suggested that spontaneous fusion occurred in cultures of adult NSCs at low frequency, which, over time, (a) could change the genetic composition and (b) might influence the behavior of NSC cultures. To unambiguously identify fusion events of NSC, we plated NSCs constitutively expressing either green fluorescent protein (GFP) or red fluorescent protein (RFP) under the control of the chicken β-actin promoter together in a single well [7]. This approach allowed us to screen for fusion events of large-cell numbers using fluorescence-activated cell sorting (FACS) without the need to depend on post hoc identification methods such as immunocytochemistry. We found that, irrespective of the culturing method, approximately 0.2% of mouse and rat NSCs coexpressed GFP and RFP, thus indicating cellular fusion. However, fused cells did not divide and could not be propagated, and died shortly after the fusion event. Surprisingly, we found that neither neurospheres nor monolayers grew within clonal boundaries, but formed heterogeneous spheres or monolayer areas of GFP- and RFP-expressing NSCs, even under low-density conditions. The data presented here show that, under standard culture conditions, NSC fusion is a rare event that does not crucially alter the cellular composition of adult NSC cultures. However, given the heterogeneous growth of NSC clones, precise and accurate culture conditions should be used when determining the behavior and potency of NSCs in vitro.

MATERIALS AND METHODS

Neural Stem Cell Isolation and Growth

The hippocampal NSCs (HCNs) isolated from the adult rat brain that we used in this study were described earlier [8]. NSCs from the adult murine whole brain were isolated as described earlier [4, 6]. Briefly, either the whole brain or the isolated hippocampus was chopped into 1-mm³ pieces and incubated in a *p*-phenylenediamine (PPD; containing 2.5 units/ml papaine) solution for 30 minutes. The lysate was mixed with an equal volume of Dulbecco's modified Eagle's medium (DMEM):F12 (containing 1 mM L-glutamine and 10% fetal bovine serum [FBS]) and filtered through a 70-μm nylon mesh. The cell suspension was mixed with an equal amount of Percoll solution and spun down (20,000*g* for 30 minutes). The cellular fraction was harvested approximately 5 ml above the erythrocytic cell layer and plated in DMEM:F12 plus 1 mM L-glutamine, 1× penicillin-streptomycin-fungizone, and 1× N2 supplement (containing 20 ng/ml fibroblast growth factor [FGF]-2 for rat NSCs,
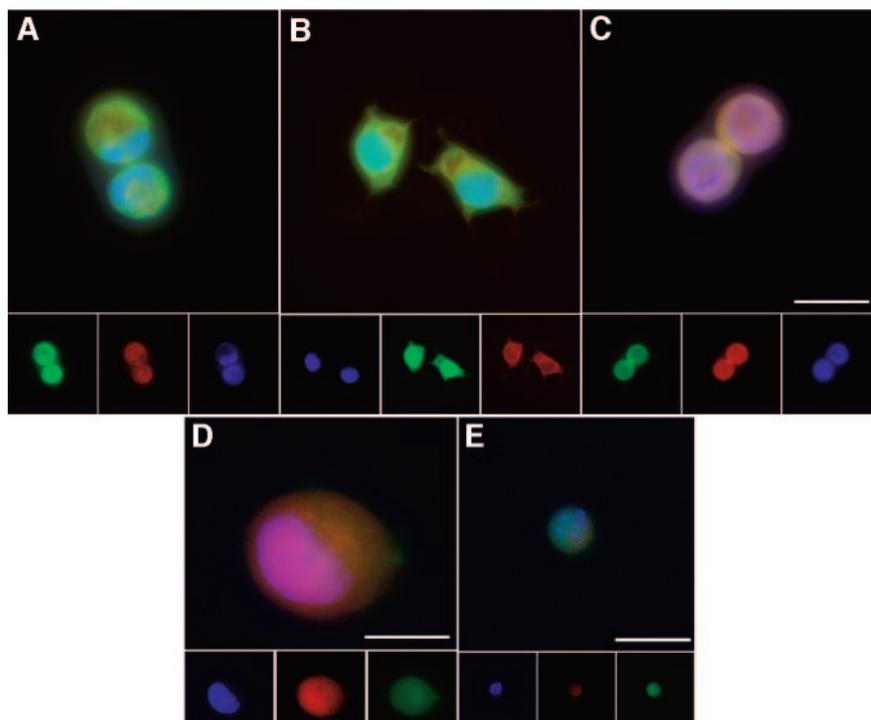
Figure 1. Spontaneous fusion of mouse and rat adult neural stem cells in vitro. **(A–C):** Three examples of fused rat NSCs. **(D, E):** Examples from mouse NSCs that were grown as monolayers under proliferating conditions. Note the distinct intracellular localization of red fluorescent protein (RFP) and green fluorescent protein (GFP), clearly demonstrating the true coexpression of the two transgenes in the same cell. Fused cells commonly showed condensed DNA, indicating the abnormal state of the cells. The large images are merged depictions of the separate channels for RFP (red), GFP (green), and 4′-6-diamidino-2-phenylindole (blue; single channels are shown in the small images). Scale bar = 10 μm in **(C, E)** and 5 μm in **(D)**.

or 20 ng/ml FGF-2, 20 ng/ml epidermal growth factor [EGF], plus or minus 5 μg/ml heparin for mouse NSCs). Rat NSCs were grown on polyornithine (PORN) and laminin coated plates; mouse NSCs were plated on uncoated tissue culture plates. At approximately 80%–90% confluency (for monolayers) or after 5 days (for neurospheres), cells were trypsinized, and a 10th of the plate was propagated.

Retroviral Transduction and Coculture of RFP- and GFP-Expressing NSCs

At passage six (mouse) or passage eight (rat), 1×10^6 NSCs were transduced with approximately 2×10^8 infectious particles of retroviruses expressing either GFP or RFP under the chicken β-actin promoter [7]. The preparation of the retrovirus was performed as described earlier [7]. After 4 days under proliferating conditions, transduced NSCs were sorted using FACS to obtain pure populations of either GFP- or RFP-expressing NSCs.

GFP- and RFP-expressing cells were plated at a 1:1 ratio at high-density (100 cells/μl) or low-density (5 cells/μl) conditions. FACS analyses were performed after 4 days of growth under proliferating conditions. To identify potential fusion events after the induction of neuronal differentiation, cells were plated for 1 day in medium containing FGF-2 (rat), or FGF-2, EGF, and heparin (mouse) and then transferred to medium containing 1 μM retinoic acid (RA) and 5 μM forskolin (FSK) for rat NSCs and 5 μM FSK plus 0.5% FBS for mouse NSCs.

Fluorescence-Activated Cell Sorting

For each condition (monolayer or neurosphere growth) and species (rat or mouse), we performed at least three independent FACS experiments of separate cell populations using a BD FACSvantage Diva system (BD Biosciences, San Diego, http://www.bdbiosciences.com). GFP was excited with a 488-nm water-cooled argon laser; RFP was excited with a 561-nm solid-state laser. The filters used were 530/30 nm for GFP and 630/22 nm for RFP. GFP- and RFP-coexpressing cells were collected in DMEM:F12 medium containing FGF-2 (rat) or FGF-2, EGF, plus or minus heparin (mouse). Cells were either immediately fixed and analyzed using a confocal microscope or pulsed with bromodeoxyuridine (BrdU).

BrdU Labeling and Immunocytochemistry

To analyze the proliferative capacity of fused NCSs, we plated GFP-/RFP-coexpressing cells on 4-well glass chamber slides (coated with PORN-laminin for rat NSCs or uncoated for mouse NSCs). Either 4 or 24 hours after FACS, 5 μM BrdU (Sigma-Aldrich, St. Louis, http://www.sigmaaldrich.com) was added into the medium. One hour after the addition of BrdU, cells were fixed (4% paraformaldehyde in 0.1 M PO$_4$ buffer for 20 minutes at 4°C) and nuclei were counterstained with 4′-6-diamidino-2-phenylindole (DAPI). To detect BrdU incorporation, cells were treated with 1 M HCl (30 minutes at 37°C) followed by 10 minutes in 0.1 M borate buffer, and then extensively washed with tris-buffered saline (TBS). Cells were then incubated for 24 hours with the primary antibody at 4°C (rat anti-BrdU; (Harlan Sera-Lab, Loughborough, United Kingdom, http://www.harlaneurope.com; 1:500 in TBS containing 3% donkey serum and 0.01% Triton X-100). A Cy5-conjugated anti-rat IgG (Jackson ImmunoResearch, West Grove, PA, http://www.jacksonimmuno.com; 1:250) was used (2 hours at room temperature) as secondary antibody. BrdU labeling and the endogenous GFP and RFP signals for all other images were analyzed after fixation of the cells and nuclear counterstaining with DAPI using an inverted confocal microscope (Bio-Rad Radiance 2100; Bio-Rad, Hercules, CA, http://www.bio-rad.com). Only general contrast enhancements and color adjustments were carried out using Adobe Photoshop 7.0 for Mac OsX (Adobe Systems, San Jose, http://www.adobe.com).

RESULTS

Spontaneous Fusion of Mouse and Rat Adult NSCs

We isolated adult NSCs from whole mouse brain or the rat hippocampus as described earlier [4]. At passage six (mouse) or passage eight (rat), we transduced NSCs with a retrovirus coding for either GFP or RFP under the chicken β-actin promoter. After 4 days under proliferating conditions, cells were sorted for the expression of GFP or RFP. For subsequent fusion analyses, we used pure populations of either RFP- or GFP-expressing adult NSCs. RFP- and GFP-expressing cells were cocultured at two different densities (high density of approximately 100 cells/μl or low density of approximately 5 cells/μl in suspen-
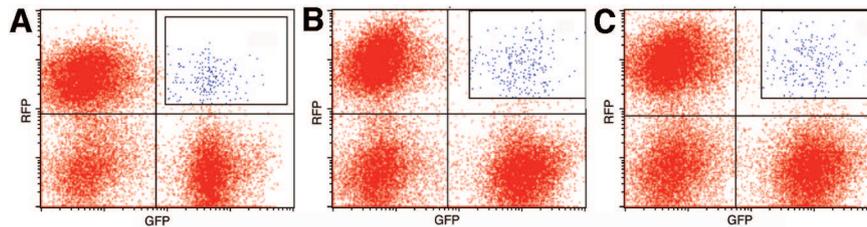
Figure 2. Fusion frequency of adult NSCs in vitro. Fluorescence-activated cell sorting (FACS) analyses reveal low but consistent fusion frequency of adult neural stem cells isolated from rat hippocampus (**A**) and mouse whole brain (**B, C**). Shown are examples of FACS experiments from NSCs grown as monolayers (**A, C**) or neurospheres (**B**). GFP- and RFP-expressing cells are depicted in blue. Note that, even though the experiments were started with pure populations expressing either GFP or RFP, FACS revealed a nonfluorescent cell population that may result from silencing of the transgene or very low levels of GFP or RFP, respectively. Abbreviations: GFP, green fluorescent protein; RFP, red fluorescent protein.

sion) under proliferating conditions. After 4 days of coculturing, RFP- and GFP-expressing adult NSCs, we performed FACS to identify RFP-/GFP-coexpressing cells. Using FACS, we found that, independent of the initial plating density, approximately 0.2% (within a range from 0.1% to 0.5%) of NSCs coexpressed RFP and GFP, indicating that an earlier fusion event had occurred (Figs. 1, 2). Figure 1 shows examples of fused NSCs derived from rat (Fig. 1A–1C) or mouse (Fig. 1D, 1E) brain that were grown for 4 days under high-density conditions. Note the distinct localization of GFP and the monomeric RFP within the cells, illustrating the actual coexpression of the two transgenes in the same cell.

We also examined rat and mouse NSCs grown as monolayers for the occurrence of fusion events after the induction of neuronal differentiation with RA and FSK. When we analyzed NSC monolayers 4 days after the addition of RA and FSK, we did not find any neuronal cells that coexpressed RFP and GFP (data not shown), suggesting that cells that fused while cycling do not survive.

Fused NSCs Do Not Divide and Cannot Be Propagated

After FACS, we attempted to propagate GFP-/RFP-expressing cells, but were unable to grow fused cells for more than 24 hours. At that time, many pyknotic cells appeared in the dish, suggesting the death of fused cells. Whereas approximately 30% of control cells incorporated the thymidine analogue BrdU 4 and 24 hours after FACS (not shown), fused cells did not pick up BrdU, indicating that none of the GFP-/RFP-expressing cells were in S-phase at any point after FACS. Because of the lack of continuous growth of fused cells, we were not able to perform chromosomal spreads to characterize the chromosomal content of fused NSCs.

NSC-Derived Neurospheres and Monolayers Grow Nonclonally

While performing the experiments to analyze the fusion frequency of NSCs, we noticed the heterogeneous composition of GFP- and RFP-expressing cells in single neurospheres (mouse NSCs) and monolayer cultures (mouse and rat NSCs; Fig. 3) under coculture conditions of NSCs expressing RFP and GFP. After 4 days of coculture, there were only very few spheres left that expressed GFP or RFP alone (Fig. 3B). This observation was true even when we cocultured RFP- and GFP-expressing NSCs at a low density of 5 cells/μl medium. As early as 24 hours after plating at low density, sphere-initiating cell clusters contained a mixed population of GFP- and RFP-expressing cells (Fig. 3C). Four days after plating the cells at low density, most of the spheres contained GFP- and RFP-expressing cells (Fig. 3E). As seen with the NSCs growing as spheres, we also found that monolayer cultures
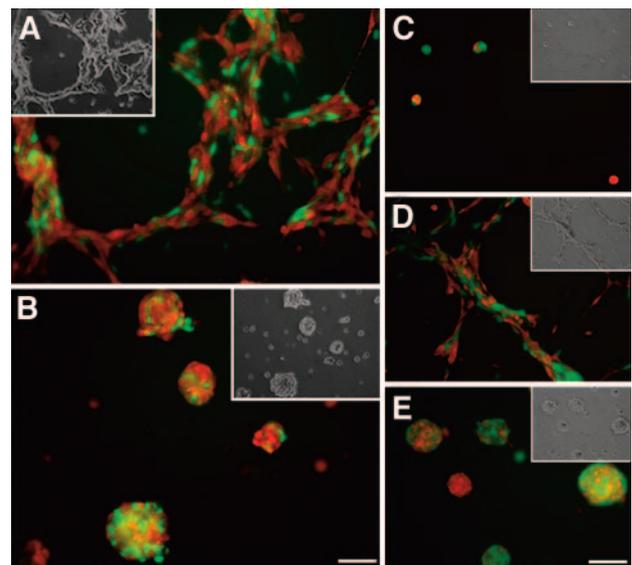
Figure 3. Adult neural stem cells grow irrespective of clonal boundaries. Shown are examples of mouse NSCs grown as monolayers (**A, D**) or neurospheres (**B, C, E**) under proliferating conditions and either plated at either high (**A, B**) or low density (**C, D, E**). Note the patched pattern of red fluorescent protein (RFP)- and green fluorescent protein (GFP)-expressing cells in the vast majority of monolayer areas and neurospheres. As early as 24 hours after plating mouse NSCs at low density, the first chimeric cell aggregates could be observed (**C**). Four days after plating NSCs at low density, monolayers had already grown in mixed areas of RFP- and GFP-expressing cells, and only very few neurospheres consisted of cells expressing either RFP or GFP alone (**E**). Insets in (**A–E**) show bright-field images of the areas analyzed. Scale bar = 100 μm (**B, E**).

showed mixed growth of GFP- and RFP-expressing cells irrespective of the initial plating density (Fig. 3A [high-density], 3D [low-density]).

DISCUSSION

We report two major findings: (a) adult NSCs fuse at a low frequency without dramatically changing the genetic composition of adult NSC cultures, because the fused cells die soon after fusion; and (b) neurospheres and monolayer cultures of NSCs do not respect clonal boundaries and grow as chimerae.

The fact that NSCs have the potential to fuse in vitro has several important implications. Cell fusion may change the genetic composition of cultures and, thus, might lead—over a prolonged culture period—to altered cellular composition of NSC cultures. The potential genetic plasticity of NSC cultures resulting from cellular fusion could, therefore, dramatically change the behavior of NSCs over time. Even

though NSCs grown in cultures might change over time [9], this change is apparently not caused by cell fusion, as we found spontaneous fusion events only at a very low frequency and fused cells died quickly. However, we may have underestimated the fusion frequency determined by GFP and RFP coexpression because fusion events between GFP- and RFP-expressing cells among themselves could not be detected with our approach. The fusion of NSCs in vitro has been described earlier [10]. Chen et al. [10] cocultured NSCs from a Cre-expressing animal with NSCs derived from a Rosa-LacZ reporter mouse to identify fused cells by β-galactosidase staining. Therefore, the identification of fused cells required the post hoc staining for β-galactosidase, which might result in a certain degree of nonspecificity because of reporter leakage or X-Gal retention. In contrast to the situation in rat and mouse NSC monolayers, Chen et al. [3, 11] found a relatively high degree of aneuploidy in astrocyte monolayers derived from the subventricular zone. These cells have been previously shown to possess NSC characteristics in vitro. However, aneuploidy does not necessarily mean that cells have fused, because aneuploidy may also occur if chromosomes are not properly segregated during mitosis.

Nevertheless, there is strong evidence that cell fusion is a rather frequent event in other systems of progenitor/stem cell cultures such as embryonic stem cell (ES) cultures. Such fusion may account for the observed "transdifferentiation" (i.e., the differentiation from somatic progenitor/stem cells from a certain tissue into a different tissue cell type) [12]. Therefore, it is important to measure the fusion frequency in other stem cell/progenitor cultures such as adult NSC cultures. With our large-scale approach—screening approximately 1.5×10^4 to 1.5×10^6 cells at a time—we also confirmed our earlier findings that the transdifferentiation of adult NSCs into endothelial cells appears not to be caused by cellular fusion [11]. Unfortunately, the karyotype of fused cells identified by RFP and GFP coexpression could not be determined in our current study because fused cells did not continue to divide, which is a prerequisite for chromosomal spreads during metaphase. However, the clear coexpression of the GFP and RFP transgenes in mouse and rat NSCs using the two most commonly used culture techniques reliably demonstrated that fusion of NSCs under standard culture conditions is rare, promptly leading to the elimination of fused cells. Therefore, the behavior of NSC cultures under standard culture conditions is not or is only marginally influenced by the occurrence of NSC fusion.

While characterizing the fusion potential of NSCs, we were surprised to find that a large fraction of neurospheres consisted of a mixed population of RFP- and GFP-expressing cells. This finding suggests the chimeric growth of neurospheres rather than the clonal expansion of single sphere-forming cells. The apparent nonclonal growth of NSCs in vitro has several important implications. The neurosphere assay is commonly used to assess stem cell properties such as self-renewal and differentiation potential [13, 14]. In fact, a recent publication [15] raised concern about the cellular composition of a neurosphere and suggested that the number of true stem cells in a single sphere is very low. Our data extend these concerns and call for caution when using the neurosphere assay as a quantitative measure to assess stem cell properties. If neurospheres are composed of cells that originated from different clones, it would be difficult to use the size of a neurosphere as a measure of proliferative capacity unless the experiment was started using single cells in a mini-well. In fact, the growth of neurospheres may be strongly influenced by the capability of free-floating cells to form aggregates, thus increasing the size of forming spheres.

CONCLUSION

In summary, we showed herein that fusion of NSCs is a rare event when these cells are cultured in vitro. Fused cells do not survive and, thus, do not significantly change the genetic composition of NSC cultures. However, we provide evidence that growing neurospheres do not respect clonal boundaries; the vast majority of neurospheres consisted of a mixed cell population of GFP- and RFP-expressing cells. Therefore, attention should be paid when interpreting the results of growth, potency, and proliferative activity of NSC cultures unless the experiments were started with single cells in one mini-well.

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DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicate no potential conflicts of interest.

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