Defining the actual sensitivity and specificity of the neurosphere assay in stem cell biology

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For more than a decade the 'neurosphere assay' has been used to define and measure neural stem cell (NSC) behavior, with similar assays now used in other organ systems and in cancer. We asked whether neurospheres are clonal structures whose diameter, number and composition accurately reflect the proliferation, self-renewal and multipotency of a single founding NSC. Using time-lapse video microscopy, coculture experiments with genetically labeled cells, and analysis of the volume of spheres, we observed that neurospheres are highly motile structures prone to fuse even under ostensibly 'clonal' culture conditions. Chimeric neurospheres were prevalent independent of ages, species and neural structures. Thus, the intrinsic dynamic of neurospheres, as conventionally assayed, introduces confounders. More accurate conditions (for example, plating a single cell per miniwell) will be crucial for assessing clonality, number and fate of stem cells. These cautions probably have implications for the use of 'cytospheres' as an assay in other organ systems and with other cell types, both normal and neoplastic.

Neurospheres—the colloquial name for a common and long-noted tissue-culture phenomenon—represent three-dimensional floating spheroid cell clusters that form *in vitro* when mitotic cells from the developing and/or adult mammalian central nervous system (CNS; including mitotically capable cells exposed to mitogens) are placed in a serum-free medium on a nonadhesive substrate. Initially developed for NSCs[1], the quantification and characterization of floating aggregates of cells has come to be used in many fields and organ systems to define and measure stem cell–like behavior. Whether derived from the nervous system[1–21], retina[8], pancreas[22], skin[23], breast[24] or bone marrow mesenchyme, these structures have been variously called 'neurospheres', 'oligospheres', 'mammospheres', 'mesospheres' and others. Recently, the newly emerging cancer stem cell field has also started to exploit free-floating spheres[25].

Over the years, the presence of neurospheres has been used as a colony-forming assay to determine whether a cell is 'stem-like' or not; the number of neurospheres to assess and compare the ability of different regions and/or ages to yield stem cells (for example, to be 'neurogenic'); the presence of cells within a single neurosphere to imply clonality; the composition of the neurosphere to indicate lineage commitment within a clone; the diameter of a neurosphere to gauge the mitogenic potency of various molecules. Clonality is the heart of stem cell biology because it is the only way to determine the true potential range and self-renewal capacity of a given individual cell[26,27]. Anything that undermines the credibility of clonal analysis undercuts identification and understanding of the properties of a putative stem cell. Therefore, in the present study, despite widely held assumptions, we asked whether neurospheres are, indeed, clonal structures; whether the sphere diameter accurately reflects the proliferative activity of NSC clones; whether the presence of multiple neural phenotypes within a sphere substantiates multipotency of a single founding cell; and whether the number of neurospheres and their repeated formation after multiple passages ('secondary neurospheres') are sufficient to affirm self-renewal.

For our studies we initially chose the optimal conditions for neurosphere formation and behavior (e.g., using young neural tissue from mouse); we subsequently validated our analysis in and extended it to older animals (e.g., adult), in tissue from multiple CNS regions (cortex, subventricular zone, striatum, ventral midbrain, spinal cord), and in multiple other species (e.g., rat, human). We provide evidence that neurospheres are motile and they merge with each other under culture conditions commonly used for the neurosphere assay, and that they therefore cannot be considered as clonal entities.

RESULTS

Culture conditions and characterization of neurospheres

Using standard techniques now well established and often used in the literature, we generated neurosphere cultures from the forebrains of newborn C57BL/6 mice[28]: Single dissociated neural cells, plated at systematically varied densities (ranging from 1×10^5 to as dilute as 1×10^2 cells/cm^2), were proliferated for 7 d in the presence of the mitogens basic fibroblast growth factor (bFGF) and epidermal growth factor (EGF; **Fig. 1a**). Spheres were harvested, re-dissociated into single cells, and re-plated in these mitogens,

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RECEIVED 7 DECEMBER 2005; ACCEPTED 9 AUGUST 2006; PUBLISHED ONLINE 21 SEPTEMBER 2006; DOI:10.1038/NMETH926

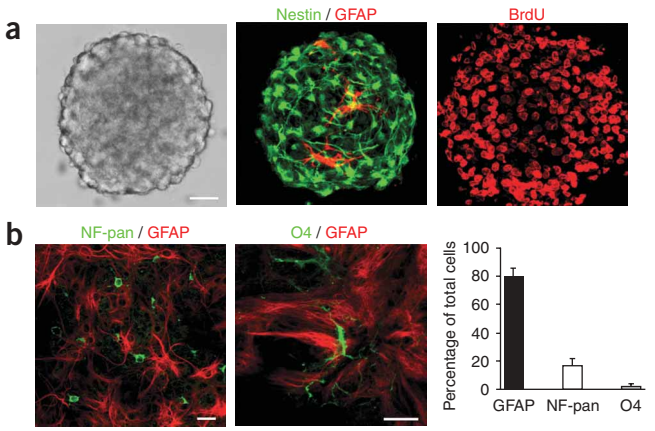

Figure 1 | Characterization of typical neurospheres isolated from the postnatal mouse forebrain and expanded in bFGF and EGF. (**a**) Micrographs (phase and confocal) of representative 'secondary' neurospheres (passaged at least once after isolation from the mouse brain). The majority of neurosphere cells express the NSC marker nestin, whereas GFAP was restricted to only a few cells. Cumulative labeling (3 d) with BrdU identifies proliferative cells. (Immunostaining for another marker of proliferation, Ki-67, is shown and quantified in **Supplementary Note**.) (**b**) Differentiation of neurospheres gives rise to astrocytes (GFAP$^+$), neurons (NF$^+$) and oligodendrocytes (O4$^+$) in the proportions indicated in the histogram (mean ± s.d.; $n = 3$). Scale bar, 25 μm.

leading to the generation of 'secondary spheres'. Repeating this procedure yielded higher-passaged neurospheres (up to eight passages studied). At all passages, nestin, a marker for immature neuroepithelial cells, was expressed in most of the neurosphere cells (**Fig. 1a**). Incorporation of 5-bromo-deoxyuridine (BrdU), a thymidine analog, confirmed proliferative activity in most of the cells (**Fig. 1a**). Differentiation of the neurospheres by growth factor withdrawal and application of serum for 5–7 d yielded a typical profile: 80% glial fibrillary acidic protein (GFAP)-immunopositive cells (presumably astrocytes), 17% neurofilament (NF)-immunopositive cells (presumably neurons), and 1–3% O4$^+$ cells (presumably oligodendrocytes; **Fig. 1b**).

Time-lapse video microscopy of neurosphere growth

We observed secondary spheres by continuous time-lapse video microscopy while they were in their undifferentiated proliferative state. Whether the cells were maintained at a conventional or at a dilute ('clonal'[5,6,12–19]) density, the neurospheres—over as little as 0.5–3 h—not only moved (in the absence of agitation or manipulation), but were drawn to each other, one inevitably absorbing the other, creating a single, larger sphere (**Fig. 2**, **Table 1** and **Supplementary Videos 1** and **2** online). Free-floating neurospheres were actually highly dynamic structures, capable of intrinsic, spontaneous locomotion, propelled in part by tiny beating cellular processes on the surface of the sphere (**Supplementary Video 3** and **Supplementary Fig. 1** online; ref. 29). 'Fusions' of neurospheres were common, rapid, multiple events regardless of whether the culture contained primary spheres or secondary, more highly passaged spheres (passages 1–8), suggesting that such behavior was not simply attributable to culture conditions—duration, passage number or other aberrations[30]. 'Merging' spheres seemed to be independent of the tissue, age, species of origin or culture plate (6-, 24-, 96-well plates). For example, neurospheres generated from embryonic day (E)14 rat striatum and cortex (data not shown), the subventricular zones of adult mice (postnatal days 60–90 ; data not shown) or the human fetal cortex (**Supplementary Videos 3** and **4** online) evinced adsorptions that were indistinguishable from those described above. The rate of cellular proliferation alone could not adequately account for the final size of most large spheres (**Table 1** and **Supplementary Note** online). In our first experiment, to determine whether proliferation alone, under the culture conditions described, could yield such large spheres over the period studied, we transferred single proliferative spheres of 10–20 μm in diameter to individual wells for continued growth. By 7 d *in vitro*, none in isolation doubled their diameter, reinforcing the notion that proliferation alone is probably not sufficient to account for the generation of large spheres (>100 μm in diameter; refs. 12,13).

Additionally, in the time-lapse studies, we frequently observed single floating cells or cells attached to the bottom of the culture dish being absorbed and incorporated into motile spheres, further contributing to the polyclonality of spheres in suspension (**Supplementary Video 5** online). Notably, even when neurospheres adhere to a culture dish, they tend to migrate and coalesce, abrogating their ability to maintain clonal boundaries and identities unless one began with a single isolated cell in a miniwell (**Supplementary Video 6** online).

Coculture of genetically labeled neurosphere-forming cells

To confirm and obtain a more quantitative estimation of the incidence and degree of 'chimeric' neurospheres, we performed the following manipulation: neurospheres were initiated from the forebrains of two different strains of transgenic mice—one that constitutively expressed *lacZ* (*E. coli* β-galactosidase (β-gal); the ROSA26 mouse) and one that constitutively expressed enhanced green fluorescent protein (EGFP; **Fig. 3a**). We cocultured the cells

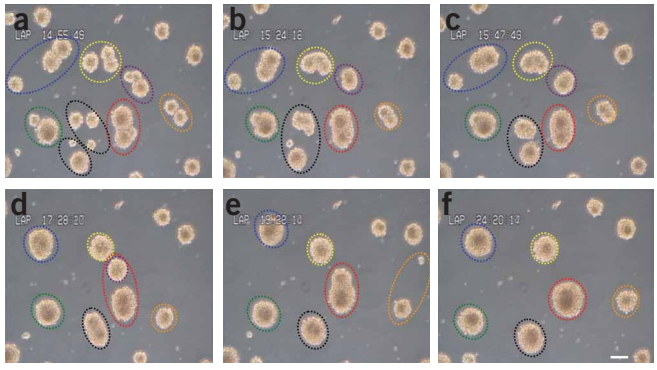

Figure 2 | Frequent, rapid and multiple 'coalescence' of secondary neurospheres. (**a**–**f**) Representative sequential frames from **Supplementary Video 1**, a time-lapse video microscopic recording, show 30 spheres at the beginning of the recording which 'merge' with each other (21 mergers counted), resulting in 10 spheres within ~10 h, and for some clusters (for example, in the upper panels) within 1 h. 'Merger' partners are circled using different colors to facilitate following their movements and changes over the 10 h of the movie. These cultures were not agitated or otherwise manipulated; the movement reflects the intrinsic locomotion of free-floating spheres. Scale bar, 100 μm.

Table 1 | Temporal and structural dynamics of neurosphere 'coalescence'

	Sphere 1 (diameter)	Sphere 2 (diameter)	After coalescence (diameter)	Duration[a] (min)
Example 1	30 μm	30 μm	40 μm	30 min
Example 2	100 μm	40 μm	120 μm	80 min
Example 3	120 μm	70 μm	150 μm	110 min

Three examples for 'coalescence-induced' neurosphere 'growth' as revealed by time-lapse video microscopy. See the **Supplementary Note** for a mathematical model. [a]Duration was determined from the first contact of two neurospheres up to complete coalescence as defined by an overall rounded border.

in a 1:1 ratio to achieve predetermined plating densities. Because it is widely believed that under very sparse suspension culture conditions neurospheres can be grown such that each sphere accurately reflects a single clone, we performed these experiments under progressively lower cell-density conditions: 'high' (1×10^5 cells/cm^2 or 500 cells/μl), 'medium' (1×10^4 cells/cm^2 or 50 cells/μl) and 'low'—often called 'clonal'—(1×10^3 cells/cm^2 or 5 cells/μl) and 'very low' (1×10^2 cells/cm^2 or 0.5 cells/μl). A plating density of up to 20 cells/μl has been considered such a 'clonal condition'[1–20]; therefore, the latter two conditions are well below that threshold. But although confocal analysis showed 'EGFP$^+$-only' spheres and 'β-gal$^+$-only' spheres, chimeric spheres composed of both EGFP$^+$ and β-gal$^+$ cells—hence 'polyclonal' spheres—were consistently present, and, indeed, constituted the majority (**Fig. 3b–e**) whether in high (96.0 ± 0.9%), medium (95.3 ± 2.0%), low 'clonal' (52.7 ± 1.3%) or even 'very low' (40.2 ± 4.5%) cell-density preparations (**Fig. 3f,g**). These findings persisted whether or not preparations were varied based on progressively increased sparseness per surface area (**Fig. 3f**) or progressively increasing dilution per volume (**Fig. 3g**). These results may differ from previous studies[5,16,18,20], because, unlike those that used only one gene to assess the homogeneity of a neurosphere population (for example, mixed cultures of GFP-expressing cells with unlabeled cells), we used two genes to affirmatively mark each cell's origin. Assessing whether GFP$^+$ neurospheres are also 'contaminated' by small numbers of non-GFP$^+$ cells, and vice versa, may not be sufficiently sensitive for the rigorous evidence of clonality. Indeed, even using two markers could underestimate the amount of coalescence, as β-gal–β-gal chimeras and EGFP-EGFP chimeras would not be distinguishable as being of mixed clonality. That such chimerism may also confound assays of human neurospheres obtained from any of a number of fetal regions (cortex, striatum, ventral midbrain, spinal cord) is suggested in **Supplementary Video 4** and **Figure 4**, where another independent marker, a species-specific antigen (human nuclear antigen (HuNu)), can be used to trace the merging of non–clonally related spheres. These observations certainly proved disconcerting for relying on neurosphere number or size as an accurate reflection of number of *bona fide* clones and proliferative capacity—even under ostensibly ideal cell-density conditions. Extrapolation of the serial dilution data in **Figure 3g** would suggest that starting with one cell per isolated well remains the only reliable guarantee of a truly clonal population. Use of the mathematical model described in **Supplementary Note** would suggest that it is also the only way to predict, with complete confidence, an increase in sphere volume based on proliferation alone.

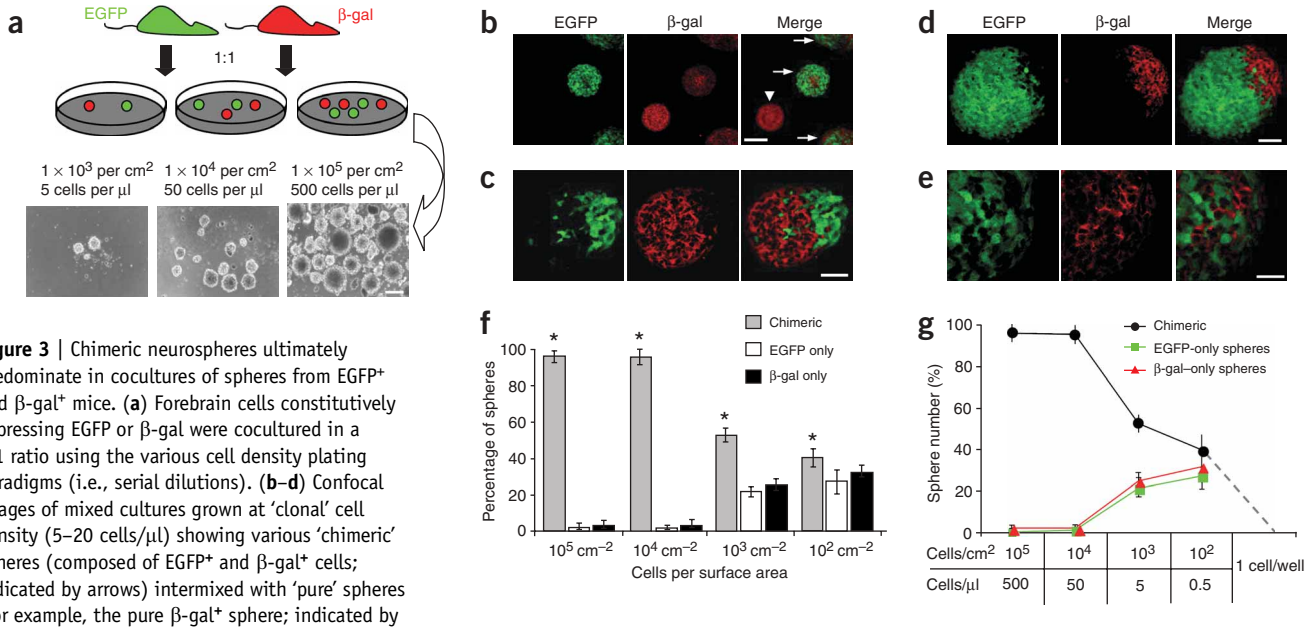

Figure 3 | Chimeric neurospheres ultimately predominate in cocultures of spheres from EGFP$^+$ and β-gal$^+$ mice. (**a**) Forebrain cells constitutively expressing EGFP or β-gal were cocultured in a 1:1 ratio using the various cell density plating paradigms (i.e., serial dilutions). (**b–d**) Confocal images of mixed cultures grown at 'clonal' cell density (5–20 cells/μl) showing various 'chimeric' spheres (composed of EGFP$^+$ and β-gal$^+$ cells; indicated by arrows) intermixed with 'pure' spheres (for example, the pure β-gal$^+$ sphere; indicated by an arrowhead). (**c**) 'Snapshot' of ongoing sphere coalescence. (**e**) Higher magnification of a chimeric sphere composed of EGFP$^+$ and β-gal$^+$ cells. (**f,g**) Composition of neurospheres grown in mixed cultures (expressed as percent of all spheres) after serial cell-density dilutions (calculated for both cells per surface area (**f**) and cells-per-volume (**g**)). *$P < 0.05$; one-way analysis of variance (ANOVA) and Tukey test. In **g**, the dotted line portion of the curve indicates extrapolation from an ultra-low density of cells (nevertheless derived from more than one mouse strain) to the condition of 'one visible cell per miniwell' ($n = 3$ independent experiments in 6-well plates; mean ± s.d.; number of secondary neurospheres is >2,000 for 'high', 'medium' and 'low' cell densities; number of secondary neurospheres is >200 for 'very low' cell density). A pure EGFP$^+$ sphere contaminated with a single β-gal$^+$ cell (and vice versa) was considered as a chimeric sphere. Scale bars in **a**,**b**, 100 μm; in **c**,**d**, 50 μm; in **e**, 25 μm.

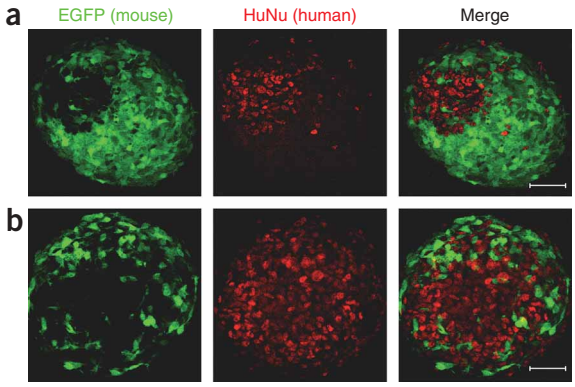

Figure 4 | Cocultured human and mouse neurospheres display chimerism. (**a**,**b**) Human cells derived from 7-week-old human fetal cortex (passage 5) labeled with human nuclei (HuNu)–specific antibody marker (red) and cocultured with mouse forebrain NSCs labeled with EGFP (green), using a final plating density of 10 cells/μl (**a**) and 20 cells/μl (**b**). Scale bars, 50 μm.

DISCUSSION

Classical tissue engineering experiments performed nearly 100 years ago with sponges showed that dispersed cells reaggregate *in vitro* to form spherical structures[31]. Thermodynamically, the shape of a sphere probably represents the most efficient cellular organization. Reaggregation and formation of one single large sphere can be achieved by constant rotation (for example, conical tubes within roller drums)[32]. The finding by others[1] that proliferative nestin-expressing neural cells grown in uncoated cell culture plates can form spheres has been used for conferring upon a cell or group of cells the most fundamental defining properties of stem cell biology. Sphere cultures are now being used across organ systems and disciplines, including the newly arising cancer stem cell field[1–25]. The results of our study show that spheres are motile and they merge, and that it is highly risky to rely solely on the sphere assay for measuring clonality, number and fate of stem cells. One must recognize the utility of the neurosphere assay while not going beyond the limits of its sensitivity and specificity; that is, not conferring upon this common tissue culture phenomenon a significance beyond that entitled by its biology. Our results indicate that sphere formation is a useful culturing tool, not a metric. Any dividing cell from virtually any tissue in serum-free medium on a nonadherent substrate will form floating cell clusters, with intercellular adhesiveness predominating. Often the appearance of the spheres from different tissues are indistinguishable, and sphere fusion also occurs in normal and neoplastic mammosphere cultures (B. Felding-Habermann, personal communication). Moreover, although intended to differentiate pluripotent embryonic stem cells, embryoid body formation is similarly mediated by coalescence (data not shown). In NSC biology, however, the ability to form spheres is an important strategy for helping to isolate and expand cells with persistent proliferative capacity, separating them from those that are postmitotic—much like a surrogate 'real-time' BrdU incorporation assay for living cells, particularly if the spheres are disaggregated frequently and allowed not to grow large. Like BrdU, it simply signals the presence of a proliferative population—a *sine qua non* of a stem cell but not the sole defining property—and, although the first step in identifying a stem/progenitor population, certainly not the end of characterization.

Compounding the problem is the considerable variability that has, over the years, crept into the variety of 'established protocols' employing the neurosphere assay (while indiscriminate reliance on its sensitivity and specificity has nevertheless increased in the literature). For instance, in 1992 investigators[1] plated 1,000 viable cells per 35 mm diameter plate (volume not specified) and measured the number of neurospheres after 6–8 d. Subsequent authors, though citing that protocol[1], have nevertheless modified it and, in so doing, introduced experimental variability from study to study, probably more consequential than assumed. This experimental variability includes duration in culture before quantification, cell density, medium volume and surface area of the culture dish[4,9,22,33]. Often protocols vary within the same research group[4,12,13,17,18]. Together, these examples suggest that the neurosphere assay is far from a well-established and adequately, uniformly calibrated assay, and may, in fact, account for some of the conflicting results obtained by different groups (a concern recently voiced editorially by one author of the previously mentioned study[34]).

In our experiments, we plated 5 cells/μl (1,000 cells/cm^2) and 0.5 cells/μl (100 cells/cm^2; cell densities that are well below the threshold used by others) into 6-well plates (2 ml), 24-well plates (0.5 ml; as per ref. 7) and 96-well plates (200 μl; B. Reynolds, personal communication). We marked cells with two independent unambiguous, permanent genetic markers (EGFP and β-gal). After 7 d, >40% of the spheres, even in the most dilute condition, were found to have elements of chimerism. Our real-time video microscopy suggests one explanation: neurospheres are constantly merging and absorbing single cells, undermining confidence that this technique alone can reliably generate clonal spheres, calling into question claims that spheres necessarily represent clonal populations and rendering as misleading the assumption that neurospheres grown in suspension accurately reflect the *in vivo* number of NSCs[11,13,16,18,19]. Experiments aimed at manipulating NSCs *in vivo* and trying to assay proliferative activity, number of NSCs or stem cell renewal using the neurosphere assay *in vitro*[10,12,13] need to be discussed again in the light of the presented findings. It could well be that *in vivo* manipulations or genetic alterations may affect neurosphere motility and fusion, two parameters not taken into account in these kind of studies to date.

The observation that spheres are dynamic structures that frequently coalesce carries several cautions: in suspension, the size and number of neurospheres as well as the variable cellular compositions may not accurately reflect the precise proliferation activity, self-renewal capacity and developmental potential of the original sphere-forming cell and its clonally related progeny. Because neurospheres should not be considered as clonal aggregates (even if 'clonal' or low cell densities are plated), and because clonality is a mainstay of stem cell biology, clonal relationships should be confirmed by adjunctive techniques: for example, (i) retroviral marking[26]; (ii) a single cell in a miniwell, recognizing that only spheres originating from that cell cultured separately from other cells can reliably be a clone[27]; or (iii) sparse, widely dispersed cells in monolayer separated by cloning rings. In some studies, the semisolid restrictive substance methylcellulose has been used to grow putative clonal neurospheres[14,15,35]. In light of our **Supplementary Video 6** in which neurospheres are noted to be motile and to merge even on coated substrates, it is concerning that published reports using methylcellulose nevertheless show neurospheres adherent to each other[14,15].

Extrapolation of our serial dilution data (**Fig. 3g**) supports the idea that plating isolated single cells in miniwells is the most rigorous method for reliably extending the use of spheres to affirm clonal relationships (i.e., beyond simply identifying proliferative cells). One should also keep in mind the observation, common in cell culture, that free-floating cells of any density have a tendency to coalesce in the center of a dish, further compromising interpretation of 'clonal' conditions (unless one starts with a single cell in that dish). Our results shown in **Table 1** and the mathematical model and test detailed in the **Supplementary Note** suggest that proliferation of a single founding cell alone cannot generate large spheres (>100 μm in diameter) after 7 d, in contrast to assumptions made in some previous reports alone[12,13]. The impossibility of generating large spheres in only 7 d is actually consistent with the earliest neurosphere reports[2,3]. It seems that, under stringent clonal conditions, a sphere of considerable size is detectable only after at least 2 weeks of proliferation. It is also important to note that a sphere is a three-dimensional structure; measuring the diameter is a two-dimensional metric. Our video-microscopy studies showed that numerous readily detectable cell divisions on the surface of the sphere resulted in subtle or no changes in the diameter because the newly generated cells were integrated into the three-dimensional structure of the sphere.

Together, our results indicate that during growth of neural cells in suspension, under conditions previously referred to as 'clonal cell density', large neurospheres are formed after 7 d only by coalescence in combination with proliferation, as also modeled mathematically. We caution that the occurrence of merging undermines the utility and rationale of the neurosphere assay as an indication of clonality. These results and cautions probably have implications for the use of 'cytospheres' as an assay with other cell types, both normal and neoplastic.

METHODS

Tissue preparation and neurosphere cultures. We initiated neurosphere cultures using published methods[1–20] on postnatal days 7–12 from (i) wild-type (C57BL/6) mice; (ii) transgenic mice (C57BL/6-TgN expressing EGFP under the control of a chicken β-actin promoter); and (iii) ROSA26 mice transgenic for *lacZ* and expressing β-gal ($n = 6$ independent preparations). We performed some experiments using neurospheres derived from adult C57BL/6 mice (postnatal days 60–90) or cortical and striatal tissues of embryonic day (E)14 Sprague-Dawley rats using standard published techniques (as well as from multiple human fetal CNS regions, see below).

We performed all preparation steps and tissue dissection in a laminar flow hood. We decapitated postnatal animals and sliced the forebrains by means of a McIllwain tissue chopper. We microdissected sections containing the subventricular zone (SVZ), digested them with trypsin, mechanically triturated them and filtered them through a sterile cell strainer (40 μm; BD Biosciences). After we performed cell counts with CASY1 TT (Schärfe System), we plated single cells in uncoated 6-well plates (2 ml; Greiner Bio-One) as well as uncoated 24-well plates (0.5 ml; Greiner Bio-One) and 96-well plates (200 μl; BD Biosciences), and proliferated them in Neurobasal medium (Invitrogen) containing 20 ng/ml bFGF (Promega), 20 ng/ml EGF (Promega) and 2 mM L-glutamine (Invitrogen; 'proliferative state'). For passaging, we collected neurospheres, centrifuged them, digested them with Accutase (Invitrogen) for 5 min at 37 °C and mechanically triturated them, and we plated single cells again after counting cells. For differentiation, we collected neurospheres with a pipette, transferred them to 24-well plates (Greiner Bio-One) and allowed them to attach on coated glass coverslips (12 mm round; poly-L-lysine, laminin; BD Biosciences). We differentiated cultures for 5–7 d in fresh Neurobasal medium in the absence of EGF and bFGF but with the subsequent addition of 10% FCS (Invitrogen) supplemented with B-27 and 2 mM L-glutamine.

For clonal experiments (1 cell per miniwell), we serially diluted cell suspensions and plated them onto 96-well plates (200 μl). We marked mini-wells containing one single cell after microscopic confirmation and analyzed neurospheres after 7 d.

We obtained human fetal neural tissue (6–12 weeks) from donations in compliance with the local ethical committee of the Faculty of Medicine, University of Freiburg, and according to the guidelines of the Network of European CNS Transplantation and Restoration (NECTAR). We isolated human neurospheres from multiple CNS regions (cortex, striatum, ventral midbrain, spinal cord). Briefly, we dissected fetal tissue under microscopic view in ice-cold sterile PBS/0.6% glucose. We incubated minced tissues in 0.1% trypsin and 0.05% DNAse for 15–20 min at 37 °C and mechanically triturated them with a fire-polished pipette into a single-cell suspension. We determined cell number and viability by the trypan exclusion method. We used proliferation and differentiation media as described above.

Cocultures and quantification. In neurosphere coculture experiments ($n = 3$ independent preparations), we mixed single cells of transgenic animals in a 1:1 ratio with final plating densities of 1×10^2 cells/cm^2 (0.5 cells/μl; 'very low'), 1×10^3 cells/cm^2 (5 cells/μl; 'low', 'clonal'), 1×10^4 cells/cm^2 (50 cells/μl; 'medium') and 1×10^5 cells/cm^2 (500 cells/μl; 'high'). We passaged neurospheres derived from each group separately, and cell plating densities at each passage were identical to those mentioned above. In addition, we cocultured mouse EGFP$^+$ cells and human NSCs derived from multiple CNS regions at a 1:1 ratio with final plating densities of 10–20 cells/μl.

For quantification, we allowed secondary spheres to attach on coated glass coverslips for 1 h and processed them for immunocytochemistry (see below). Quantification and evaluation of mixed neurospheres were performed by three different investigators blinded to the study conditions at an epifluorescence microscope (Leica DMRX) by using 20× and 40× objective lenses and appropriate filters. More than 2,000 spheres were evaluated for 'high', 'medium' and 'low' cell densities; more than 200 spheres were assessed for 'very low' cell density.

Immunocytochemistry. We fixed neurospheres and differentiated cells with 4% paraformaldehyde (PFA) for 1 h. After washings in 0.1 M PBS (pH 7.4) and blocking in 3% BSA for 1 h, we applied the following antibodies overnight in 1% BSA, 0.1% Triton X-100 and 0.1% sodium azide at 4 °C: mouse nestin-specific antibody (1:200; Chemicon), mouse pan-neurofilaments–specific antibody (NF; 1:3; Zymed), mouse O4-specific antibody (1:250; Chemicon; incubation without Triton X-100), mouse β-gal–specific antibody (1:1,000; Promega), rabbit glial fibrillary acidic protein (GFAP)–specific antibody (1:1,000; Dako), mouse human nuclei (HuNu)-specific antibody (HuNu; 1:500, Chemicon), rabbit Ki-67–specific antibody (Abcam; 1:400). To detect primary antibodies, we applied Alexa 488–conjugated goat rabbit-specific (1:500; Molecular Probes) and Cy3-conjugated goat mouse-specific (1:500;

Dianova) antibody for 4–6 h at room temperature (25 °C). In control experiments, omitting the primary antibodies did not result in specific staining.

In all experiments, we counterstained cell nuclei with Hoechst 33258 or DAPI (for 10 min, and after several rinses we coverslipped samples in Mowiol/DABCO (Calbiochem). Fluorescent signals were evaluated by means of a confocal microscope (Leica TCS NT) equipped with standard filter sets.

BrdU experiments. To detect proliferating cells based on their incorporation of BrdU, we cumulatively labeled secondary neurospheres with BrdU (10 μM; Sigma) for 3–6 d, fixed them with 4% PFA, incubated them in 2 N HCl for 30 min at 37 °C and washed them with borate buffer (pH 8.5) for 15 min. Thereafter, we applied a rat monoclonal BrdU-specific antibody (1:1,000; Harlan) and Alexa 568–copnjugated goat rat-specific antibody (1:500; Molecular Probes).

Time-lapse video microscopy. We performed time-lapse video microscopy ($n > 40$ recordings) using an inverted microscope (Leica DM IRB) equipped with a climate chamber (Leica) and connected to a color video monitor (BT-H1450Y, Panasonic) and to a videotape recorder (6730, S-VHS, Panasonic). We programmed the videotape recorder to capture images every 3.5 s. During continuous observation (up to 72 h), we kept 6-, 24- and 96-well plates containing neurosphere cultures in a humidified atmosphere at 37 °C and 5% CO_2. Movies were digitized at the Videocenter of the University of Freiburg by using commercially available software (Pinnacle Systems).

Note: Supplementary information is available on the Nature Methods website.

ACKNOWLEDGMENTS

We acknowledge the contributions of M. Ditter, C.E. Hagemeyer, A. Papazoglou, A. Klein and A. Fahrner. We thank W. Wünsch for his help digitizing the videos. We also thank J. Loring for technical help with **Supplementary Video 6** and A. Eroshkin for discussion on the mathematical model in the **Supplementary Note**. This work was supported by the Deutsche Forschungsgemeinschaft (Si874/2-1), the US National Institutes of Health, the American Parkinson's Disease Association, Children's Neurobiological Solutions, the March of Dimes, Margot Anderson Brain Restoration Foundation and Project ALS.

COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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1. Reynolds, B.A. & Weiss, S. Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system. *Science* **255**, 1707–1710 (1992).
2. Reynolds, B.A., Tetzlaff, W. & Weiss, S. A multipotent EGF-responsive striatal embryonic progenitor cell produces neurons and astrocytes. *J. Neurosci.* **12**, 4565–4574 (1992).
3. Reynolds, B.A. & Weiss, S. Clonal and population analyses demonstrate that an EGF-responsive mammalian embryonic CNS precursor is a stem cell. *Dev. Biol.* **175**, 1–13 (1996).
4. Craig, C.G. *et al. In vivo* growth factor expansion of endogenous subependymal neural precursor cell populations in the adult mouse brain. *J. Neurosci.* **16**, 2649–2658 (1996).
5. Represa, A., Shimazaki, T., Simmonds, M. & Weiss, S. EGF-responsive neural stem cells are a transient population in the developing mouse spinal cord. *Eur. J. Neurosci.* **14**, 452–462 (2001).
6. Chojnacki, A. & Weiss, S. Isolation of a novel platelet-derived growth factor-responsive precursor from the embryonic ventral forebrain. *J. Neurosci.* **24**, 10888–10899 (2004).
7. Tropepe, V. *et al.* Distinct neural stem cells proliferate in response to EGF and FGF in the developing mouse telencephalon. *Dev. Biol.* **208**, 166–188 (1999).
8. Tropepe, V. *et al.* Retinal stem cells in the adult mammalian eye. *Science* **287**, 2032–2036 (2000).
9. Seaberg, R.M. & van der Kooy, D. Adult neurogenic regions: the ventricular subependyma contains neural stem cells, but the dentate gyrus contains restricted progenitors. *J. Neurosci.* **22**, 1784–1793 (2002).
10. Ramirez-Castillejo, C. *et al.* Pigment epithelium-derived factor is a niche signal for neural stem cell renewal. *Nat. Neurosci.* **9**, 331–339 (2006).
11. Hitoshi, S. *et al.* Primitive neural stem cells from the mammalian epiblast differentiate to definitive neural stem cells under the control of Notch signalling. *Genes Dev.* **18**, 1806–1811 (2004).
12. Kippin, T.E., Martens, D.J. & van der Kooy, D. p21 loss compromises the relative quiescence of forebrain stem cell proliferation leading to exhaustion of their proliferation capacity. *Genes Dev.* **19**, 756–767 (2005).
13. Kippin, T.E., Kapur, S. & van der Kooy, D. Dopamine specifically inhibits forebrain neural stem cell proliferation, suggesting a novel effect of antipsychotic drugs. *J. Neurosci.* **25**, 5815–5823 (2005).
14. Kukekov, V.G., Laywell, E.D., Thomas, L.B. & Steindler, D.A. A nestin-negativ precursor cell from the adult mouse brain gives rise to neurons and glia. *Glia* **21**, 399–407 (1997).
15. Suslov, O.N., Kukekov, V.G., Ignatova, T.N. & Steindler, D.A. Neural stem cell heterogeneity demonstrated by molecular phenotyping of clonal neurospheres. *Proc. Natl. Acad. Sci. USA* **99**, 14506–14511 (2002).
16. Imura, T., Kornblum, H.I. & Sofroniew, M.V. The predominant neural stem cell isolated from postnatal and adult forebrain but not early embryonic forebrain expresses GFAP. *J. Neurosci.* **23**, 2824–2832 (2003).
17. Kim, M. & Morshead, C.M. Distinct populations of forebrain neural stem and progenitor cells can be isolated using side-population analysis. *J. Neurosci.* **23**, 10703–10709 (2003).
18. Morshead, C.M., Garcia, A.D., Sofroniew, M.V. & van der Kooy, D. The ablation of glial fibrillary acidic protein-positive cells from the adult central nervous system results in the loss of forebrain neural stem cells but not retinal stem cells. *Eur. J. Neurosci.* **18**, 76–84 (2003).
19. Molofsky, A.V. *et al.* Bmi-1 dependence distinguishes neural stem cell self-renewal from progenitor proliferation. *Nature* **425**, 962–967 (2003).
20. Nunes, M.C. *et al.* Identification and isolation of multipotential neural progenitor cells from the subcortical white matter of the adult human brain. *Nat. Med.* **9**, 439–447 (2003).
21. Zhang, S.C., Lipsitz, D. & Duncan, I.D. Self-renewing canine oligodendroglial progenitor expanded as oligospheres. *J. Neurosci. Res.* **54**, 181–190 (1998).
22. Seaberg, R.M. *et al.* Clonal identification of multipotent precursors from adult mouse pancreas that generate neural and pancreatic lineages. *Nat. Biotechnol.* **22**, 1115–1124 (2004).
23. Toma, J.G. *et al.* Isolation of multipotent adult stem cells from the dermis of mammalian skin. *Nat. Cell Biol.* **3**, 778–784 (2001).
24. Matsuda, M. *et al.* Serotonin regulates mammary gland development via an autocrine-paracrine loop. *Dev. Cell* **6**, 193–203 (2004).
25. Hemmati, H.D. *et al.* Cancerous stem cells can arise from pediatric brain tumors. *Proc. Natl. Acad. Sci. USA* **100**, 15178–15183 (2003).
26. Snyder, E.Y. *et al.* Multipotent neural cell lines can engraft and participate in development of mouse cerebellum. *Cell* **68**, 33–51 (1992).
27. Parker, M.A. *et al.* Expression profile of an operationally-defined neural stem cell clone. *Exp. Neurol.* **194**, 320–332 (2005).
28. Gritti, A. *et al.* Multipotent neural stem cells reside into the rostral extension and olfactory bulb of adult rodents. *J. Neurosci.* **22**, 437–445 (2002).
29. Lobo, M.V. *et al.* Cellular characterization of epidermal growth factor-expanded free-floating neurospheres. *J. Histochem. Cytochem.* **51**, 89–103 (2003).
30. Morshead, C.M., Benveniste, P., Iscove, N.N. & van der Kooy, D. Hematopoietic competence is a rare property of neural stem cells that may depend on genetic and epigenetic alterations. *Nat. Med.* **8**, 268–273 (2002).
31. Wilson, H.V. On some phenomena of coalescence and regeneration in sponges. *J. Exp. Zool.* **5**, 245–258 (1907).
32. Studer, L., Tabar, V. & McKay, R.D.G. Transplantation of expanded mesencephalic precursors leads to recovery in parkinsonian rats. *Nat. Neurosci.* **1**, 290–295 (1998).
33. Garcia, A.D., Doan, N.B., Imura, T., Bush, T.G. & Sofroniew, M.V. GFAP-expressing progenitors are the principal source of constitutive neurogenesis in adult mouse forebrain. *Nat. Neurosci.* **7**, 1233–1241 (2004).
34. Reynolds, B.A. & Rietze, R.L. Neural stem cells and neurospheres – re-evaluating the relationship. *Nat. Methods* **2**, 333–336 (2005).
35. Gritti, A. *et al.* Epidermal and fibroblast growth factors behave as mitogenic regulators for a single multipotent stem cell-like population from the subventricular region of the adult mouse forebrain. *J. Neurosci.* **19**, 3287–3297 (1999).