Stem and progenitor cells: the premature desertion of rigorous definitions

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A current disturbing trend in stem cell biology is the abandonment of rigorous definitions of stem and progenitor cells in favor of more ambiguous, all-encompassing concepts. However, recent studies suggest that there are consistent, functional differences in the biology of these two cell types. Admittedly, it can be difficult to harmonize the *in vivo* and *in vitro* functional differences between stem and progenitor cells. Nonetheless, these distinctions between cell types should be emphasized rather than ignored, as they can be used to test specific hypotheses in neural stem cell biology.

Neural stem cells are defined on the basis of two functional properties: a seemingly unlimited capacity for self-renewal and the ability to generate multiple mature neural cell types (multipotentiality). By contrast, progenitors are proliferative cells with a limited capacity for self-renewal and are often unipotent [1,2]. Currently, many different neural cell populations are being labeled with the term 'stem cell'; indeed, some appear to use the term for any proliferating cell in the nervous system. The promiscuous use of 'stem cell' reflects a recent trend towards the abandonment of precise, classifying nomenclature for the varied and distinct cell types that exist along the cell lineage of a given tissue.

To replace the rigorous descriptions of stem cells, more vague and all-encompassing definitions have been offered. For example, some investigators have suggested that the term stem cell should 'include all self-renewing progenitor cells that have the broadest developmental potential available within a particular tissue at a particular time' [3] or, more recently, that we should abandon altogether the idea that a stem cell is a discrete cellular entity, and rather posit that 'a stem cell most accurately refers to a biological function that can be induced in many distinct types of cells, even differentiated cells' [4]. This viewpoint is captured nicely in the artwork from a recent review [4] (Fig. 1a). Part of the impetus for broadening the definition of a stem cell comes from recent reports of previously unrecognized stem cell plasticity, wherein tissue-specific stem cells have been observed to cross lineage boundaries and generate tissues derived from multiple primary germ layers [5–9].

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Neural stem cells are purported to be among the most 'plastic' of the somatic stem cells, and have been shown to contribute to all three primary germ layers when introduced into an early blastocyst [10,11]. The idea that 'anything can make anything' [12] that has emerged from these studies has not only blurred the distinction between stem cells of different tissue origins, but has also distorted (less appropriately) the distinction between stem and progenitor cells within any single tissue system. A problem with broad, formless definitions is they hint that investigators should perhaps resign themselves to never being able to draw meaningful distinctions between different cell types. This is an outlook that not only smacks of pessimism, but is also misleading. For example, in the blood system, hematopoietic stem and progenitor cells have been unambiguously characterized into distinct subpopulations based on both phenotype and function (Fig. 1b), in direct juxtaposition with the idea that a stem cell is not a discrete entity (Fig. 1a). These subpopulations of hematopoietic cells include long-term and short-term reconstituting stem cell populations, as well as lineage-restricted progenitor cells [13,14]. Recent evidence suggests that, although neural stem and progenitor cells appear to overlap somewhat in terms of gene expression, they can be clearly distinguished on the basis of differences in Notch signaling [15] and, importantly, on the basis of functional attributes (Fig. 2 and Table 1). However, as demonstrated by comparing Fig. 1b with Fig. 2, neural lineage models are surely in their early days.

Adult neurogenic regions contain distinct progenitor and stem cells: differences in biology, not in semantics

There are two well-characterized regions of the adult rodent forebrain in which neurogenesis continues throughout life: the subependymal cell layer that lies directly subjacent to the ciliated ventricular ependyma and the subgranular cell layer (SGL) in the dentate gyrus of the hippocampus. Both regions contain cell populations that proliferate *in vivo* and generate cells that ultimately mature and integrate as functional granule cell interneurons [16,17].

A recent study compared the proliferative, neurogenic cells present in these two regions, in an effort to determine which other properties were shared between the two resident cell populations [18]. This was achieved by

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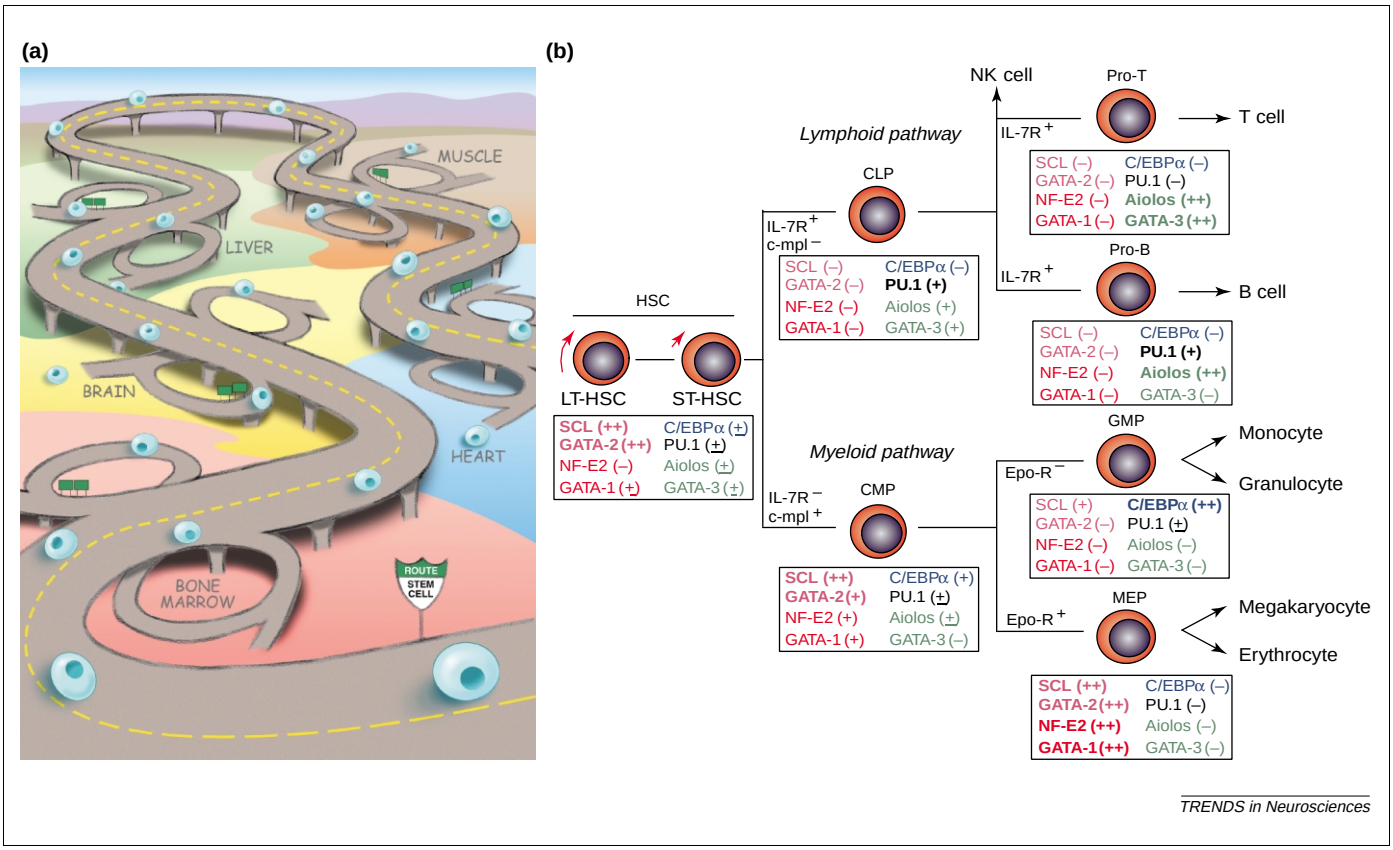

Fig. 1. Juxtaposition of opposing concepts of stem cells. (a) A depiction of adult stem cells, illustrating the recently described (although still controversial) characteristics of plasticity and diversity of tissue origin. This model suggests that the stem cells (light blue) are not tissue-specific and can reversibly enter multiple tissue compartments through the bloodstream ('Route Stem Cell' highway) and generate appropriate cell types. Reproduced, with permission, from Ref. [4]. (b) Proposed model [13] for the major differentiation pathways from hematopoietic stem cells, illustrating some of the steps in the blood stem cell lineage and highlighting the differences between stem and progenitor cells. The red arrows indicate self-renewal ability of the LT-HSC and ST-HSC. Each stage is discrete and is clearly defined by its own expression profile. Use of colored and bold text and of plus and minus symbols relates to the paper in which this figure originally appeared [13] and is not considered further in this review. Abbreviations: CLP, common lymphocyte progenitor; CMP, common myeloid progenitor; GMP, granulocyte and monocyte progenitor; LT-HSC, long-term hematopoietic stem cell; MEP, megakaryocyte and erythrocyte progenitor; Pro-B, B-cell progenitor; Pro-T, T-cell progenitor; ST-HSC, short-term hematopoietic stem cell. Part (b) reproduced, with permission, from Ref. [13], © (2000) Nature Publishing Group (http://www.nature.com).

employing a precise microdissection strategy in combination with two well-characterized *in vitro* assays: a colony-forming (neurosphere) assay [19] and a monolayer culture assay [20]. These experiments revealed that functionally different cell types (i.e. stem and progenitor cells) are responsible for the generation of neurons in the adult subependymal layer and hippocampal SGL.

Briefly, it was shown that in the case of the proliferative cells resident within the hippocampal SGL, two different progenitor types exist: one that generates exclusively neuronal progeny and another that generates exclusively glial progeny. There were no cases of single proliferative cells generating both neural cell types [18]. Further, neither of these progenitors was capable of extensive self-renewal: dissociation of primary colonies into single cells never resulted in the generation of secondary progenitor colonies. In summary, these two cell types are not multipotential and are only capable of very limited self-renewal; hence, they best fit the aforementioned description of relatively restricted progenitor cells. By contrast, individual proliferative cells resident within the subependymal tissue that surrounds the hippocampus (Fig. 3) are capable both of generating neurons, astrocytes and oligodendrocytes and of long-term self-renewal; thus, these cells best fit the definition of a stem cell [2,18,21].

The empirical classification as neural stem or progenitor cell can generalize across experimental techniques and provide insight into neurogenic mechanisms

Importantly, the functional classification of the cells isolated from the hippocampal SGL as progenitors and from the ventricular subependyma as stem cells depended on the accurate dissection of specific tissues, and not on the culture method (monolayer versus neurosphere), mitogens or rodent species used. It has been suggested elsewhere [22] that these latter methodological variables play a crucial role in what is defined as a neural stem cell. However, the fact that the results of this recent comparative study were robust enough to generalize across these variations in technique underscores the utility of separate functional definitions for progenitor and stem cells.

The aforementioned study [18] challenges the widely held assumption that stem cells are resident in the hippocampal SGL and are responsible for the generation of neurons that occurs there. This assumption was based on the results of older culture studies of the whole,

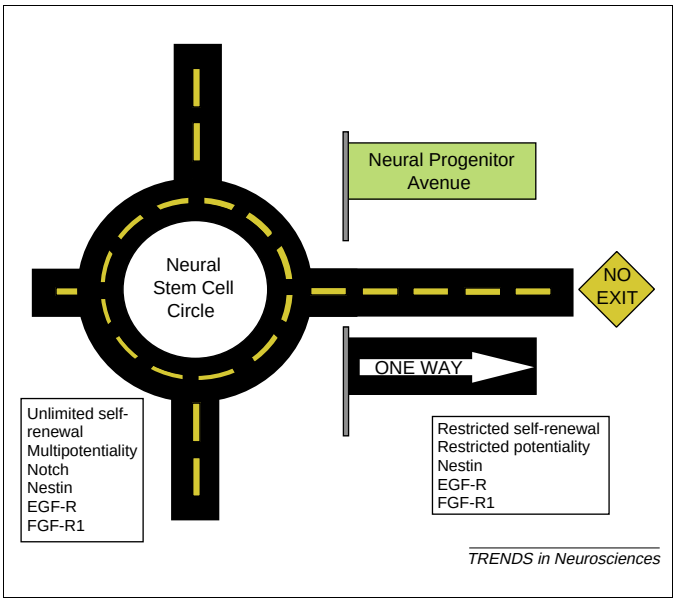

Fig. 2. A tongue-in-cheek model (with apologies to H. Blau) for neural stem and progenitor cells that emphasizes the empirically testable differences between stem and progenitor cells. The roundabout Neural Stem Cell Circle is a metaphor for the two cardinal properties of stem cells: the stem cell can self-renew (continue around the roundabout) and also asymmetrically generate neural progenitor cells that can differentiate into several different neural lineages (streets). For example, neural progenitor cells can exit from Neural Stem Cell Circle and proceed along Neural Progenitor Avenue, which is a one-way street that ends in a cul-de-sac (i.e. these cells are restricted in their ability to self-renew and in their options for differentiation). The boxes indicate functional properties and expression profiles for neural stem cells (left) and progenitor cells (right).

unfractionated hippocampus [20,23]. The more precise localization of neural stem cells to the ventricular subependyma and restricted neuronal progenitors to the hippocampal SGL [18] provides insight into the mechanisms of neuron generation in this region. The newborn adult neurons in the SGL could be generated by the neuron-specific progenitors present therein, as well as by the stem cells that exist peripherally in the ventricular subependyma around the hippocampus; these stem cells might generate progenitor cells that migrate into the dentate gyrus. Interestingly, support for both of these neuronal sources has emerged from two recent studies. The first study showed that, after brain irradiation, the genesis of neurons (but not that of glia) in the SGL was perturbed. One interpretation offered for this result was that neuron-specific progenitors resident within the SGL were the primary cell type injured by irradiation. Further, if cells from the whole hippocampal lobe were dissected, the cells present in these cultures (potentially from included subependymal tissue) retained the ability to generate multiple cell lineages *in vitro*, suggesting that the neurogenic capacity of these peripherally located stem cells was not affected by irradiation [24]. The second study demonstrated that the progeny of proliferating cells, abundant in the periventricular regions adjacent to the hippocampus, could be stimulated by ischemia and exogenous growth factors to migrate into both the CA1 and SGL regions and to generate functional neurons. This suggests an extra-SGL origin of at least some of the newly born neurons in the SGL [25].

Stem cells and progenitors have been functionally distinguished in other brain regions and neural tissues

Other studies have demonstrated consistent biological differences between neural stem and progenitor cells. Indeed, progenitor-forming and stem-cell-forming sphere colonies were distinguished functionally in one of the early studies that originally described the neurosphere assay. Up to 10% of the spheres that formed from tissue dissected at embryonic day 14 in this study did not generate all three neural cell types and were thus described as progenitor-derived spheres [26]. Oligodendroglial progenitors have also been shown to generate 'oligospheres' that have limited self-renewal capacity, and upon differentiation generate only oligodendrocytes [27]. Furthermore, in the mammalian eye there are *bona fide* retinal neural stem cells that can be clearly distinguished from neural retinal progenitor cells on the basis of rigorous functional stem cell and progenitor definitions. The stem cells can generate all retinal cell types, be passaged multiple times *in vitro*, and also be isolated from adult eyes. By contrast, the progenitor cells do not self-renew *in vitro* and have only a transient perinatal existence, as they are not present in adult eyes [28].

In direct opposition, another report has suggested that the line between neural progenitor and stem cells is indistinct. In this study, well-characterized oligodendrocyte progenitor cells from the early postnatal optic nerve were reprogrammed to grow as sphere colonies and to exhibit stem-like properties *in vitro* [29]. These reprogrammed cells exhibited initial multipotentiality and

Table 1. Summary of the functional characteristics that can be used to differentiate between neural stem and progenitor cells[a]

Characteristic	Neural stem cell	Progenitor cell
Self-renewal *in vivo*	Unlimited; for the lifetime of the organism	Limited; transient
Self-renewal *in vitro*	Unlimited; can reach maximum number of population doublings before transformation	Does not reach maximum number of population doublings
Potentiality	Multipotent (ability to generate neurons, astrocytes and oligodendrocytes)	Most often unipotent; can be multipotent
Maintenance of self-renewal and multipotentiality	Yes	No

[a]Neural stem cells exhibit the properties of unlimited self-renewal and multipotentiality. Moreover, these characteristics are present at isolation in *bona fide* neural stem cells and do not 'emerge' after long-term culture or immortalization, and they are maintained *in vivo* for the lifetime of the organism. *In vitro*, neural stem cells can maintain their multipotentiality over extended passages and can self-renew. Once transformation events occur *in vitro*, the population is different from the starting population and, thus, the cells no longer truly exhibit self-renewal. Therefore, 'unlimited' *in vitro* self-renewal can be said to occur up to the point (number of passages or population doublings) at which such transformation events begin to occur.

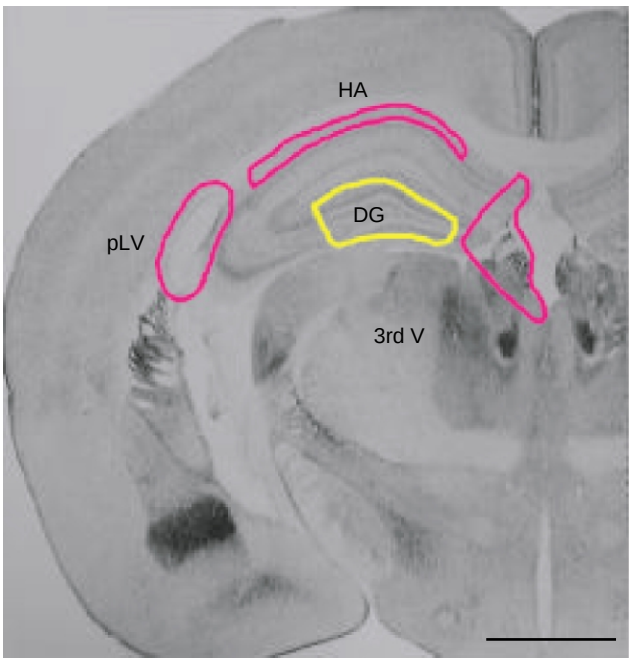

Fig. 3. Neural stem cells exist in the ventricular subependymal tissue that surrounds the hippocampus, but not within the dentate gyrus itself. The areas highlighted in pink contain subependymal cells, a small proportion of which generate multipotential, self-renewing neurospheres *in vitro*. By contrast, cultures of the dentate gyrus proper do not contain cells with these properties. Abbreviations: 3rd V, third ventricle; DG, dentate gyrus; HA, hippocampal arch; pLV, posterior lateral ventricle. Scale bar, 1 mm. Reproduced, with permission, from Ref. [18], © (2002) Society for Neuroscience.

could be passaged *in vitro* to give secondary spheres, although the maintenance of these two properties *in vitro* and the persistence of these cells *in vivo* were not assayed. Closer examination has revealed that, although there are indeed progenitors in the perinatal optic nerve (and indeed in many perinatal brain regions) that initially can be passaged and are multipotential, these characteristics are exhibited by the cells without them having to be forced through a reprogramming protocol *in vitro* [30]. Moreover, these cells quickly lose their neuronogenic capacity over time *in vitro* and their self-renewal ability over time *in vivo* and thus they are more appropriately classified as progenitors. This result underscores the importance of repeatedly assaying ongoing cultures for the two functional stem cell criteria [30] to be able to correctly classify proliferating cell types.

Stem cells and progenitors have been functionally distinguished in non-neural tissues

These findings mirror those from other, non-neural tissue systems displaying biological differences between stem and progenitor cells; for instance, there are clearly distinguishable long-term and short-term repopulating blood stem cells and progenitors in the hematopoietic system [13,14]. Additionally, slow-cycling follicular stem cells can be distinguished functionally and spatially from the fast-dividing, transit-amplifying epidermal progenitor cells that have a low capacity for self-renewal [31–35].

The stem cell uncertainty principle: stem and progenitor cells operationally defined *in vitro* and *in vivo*

Given that recent studies have brought to light the biological distinctiveness of neural stem and progenitor cells, it is timely to re-examine these definitions (Table 1), and to critically determine what their role should be in guiding new research.

Stem cells are defined in terms of their functional properties. This presents an inherent problem because such properties can be assessed only by experimental manipulation, and such manipulation can itself alter the characteristics of cells. Keeping this 'stem cell uncertainty principle' in mind, what are the functions that a cell needs to exhibit for classification as a stem cell? It is our contention that a cell must demonstrate unlimited self-renewal, multipotentiality and, crucially, the maintenance of these properties.

An operational stem cell definition in vitro

To assay for stem cell functions *in vitro*, typically the region of interest (often a region that has been shown to contain cells that divide *in vivo*) is dissected, dissociated into single cells and exposed to mitogens. This is a straightforward assay to determine whether or not cells from the region are capable of proliferation, and is especially informative when the cells are known to be quiescent *in vivo*. Neural tissue is generally grown as a monolayer on an adherent substrate or in suspension as neurospheres. The resulting cultures can then be manipulated further to assay for self-renewal and multipotentiality. For example, individual, clonally derived neurospheres can be dissociated into single cells and re-plated in mitogens to determine whether secondary neurospheres will form – that is, whether the neurosphere-initiating cell has divided symmetrically to generate copies of itself. Individual neurospheres can also be removed from mitogens and encouraged to differentiate into mature neural cell types; these can then be assayed by standard immunocytochemistry techniques for the presence of neurons, astrocytes and oligodendrocytes (i.e. multipotentiality). In the case of monolayer cultures, clonality can be established more laboriously, by retrovirally tagging a single cell and demonstrating by Southern analysis (using a single insertion site) that its progeny were derived clonally [20]. In either case, it is crucial that clonality is clearly demonstrated if meaningful insights into the biology of individual neural stem cells are to be gained.

Proliferation in vitro *is not in itself an indication of 'stemness'*

A common misconception becomes evident from the preceding description of the *in vitro* assessment of stem cells and warrants mention here. It is important to emphasize that neural cell proliferation and/or neurosphere formation are not themselves sufficient criteria for classification as a stem cell. There is a growing list of examples of neural cells that are capable of proliferation and sphere formation *in vitro* that are not stem cells. This includes ependymal cells [21], oligodendrocyte progenitors [27], neural retina progenitors [28], a subpopulation of cells from the embryonic striatum [26], transiently multipotential progenitor

cells from perinatal optic nerve, cortex, striatum, and cerebellum [30], and dentate gyrus neuron-specific and glia-specific progenitors [18]. Furthermore, long-term self-renewal ability needs to be assessed rigorously: at each of multiple passages, cells should be re-assayed for multipotentiality. A recent study that uncovered proliferative progenitor cells from diverse perinatal brain regions illustrated that, although these cells maintain their multipotentiality for the first few passages, this property is lost after a relatively short time, both *in vitro* and *in vivo* [30] – suggesting that these cells are progenitors and not stem cells. By contrast, there are *bona fide* neural stem cells capable of maintaining their multipotentiality after many passages in both the embryonic ventricular germinal zone and the adult subependyma [30,36,37].

Potential pitfalls of defining stem cells according to in vitro *criteria*

There are other problems with the *in vitro* operational definitions of stem cells. First, how much self-renewal is sufficient for a cell to be called a stem cell? Although the strict stem cell definition suggests that unlimited self-renewal is exhibited, this characteristic is perhaps more easily met *in vivo*: perpetuation of a multipotential cell population throughout the lifetime of the organism would seem to be an unambiguous criterion. Parenthetically, very few studies have examined neural stem cells in aged animals, and there is disagreement regarding whether or not there is diminution in the number of neural stem cells that can be isolated from these senescent animals [38,39]. Interestingly, even in the hematopoietic system, stem cells begin to decrease in number after several serial transplantations into different animals; this is perhaps due to the concomitant telomere shortening [40]. Nonetheless, unlimited self-renewal is a more difficult distinction to make *in vitro* because after prolonged time in culture (e.g. more than ten passages for neural stem cells) one begins to observe transformation events, as evidenced by alterations in proliferation kinetics, growth factor dependence, cell adhesion and gene expression [37]. We intentionally use the term transformation rather than immortalization in this context, as stem cells are by definition immortal, or capable of unlimited self-renewal. As such, they do not require immortalizing events to occur *in vitro* for them to continue to proliferate over extended periods of time. Thus, unlimited self-renewal *in vitro* can be operationally defined as the ability to reach the maximum number of passages or population doublings before transformation events are observed.

A second, related problem is that cell properties clearly change over time *in vitro* – which properties can a cell change and still be considered a stem cell? A recent study examined the effects of long-term culture on neurospheres and found that, although some characteristics (e.g. proliferation kinetics, growth factor dependence, cell adhesion and gene expression) changed over time *in vitro*, even after as many as 40 passages these cells retained the ability to generate neurons, astrocytes and oligodendrocytes. These long-term passaged cells also generated solid tumors when injected subcutaneously into murine hosts [37]. Because stem cells are immortal, they might be more susceptible to transformation events that not only change their properties *in vitro*, but also make them tumorigenic. According to the rigorous stem cell definitions, these would not be considered stem cells because they no longer truly meet the criterion for self-renewal. The cells that renewed after the transformation event are now different from the starting population and thus are not the same 'selves' any more.

In keeping with this line of reasoning, would any (neural) cell line that has been manipulated to proliferate *in vitro* over an extended time (e.g. one intentionally immortalized with an oncogene) meet the self-renewal criterion for stem cells? No. It is our contention that stem cell properties must be observable initially and be maintained, and not 'emerge' after prolonged culture periods or purposeful oncogenetic immortalization. If a cell type requires an immortalization event to proliferate *in vitro* for extended periods, then we suggest that this cell would be more accurately classified as a progenitor. Self-renewal (and self-maintenance) implies that a stem cell continues to show the same properties over time *in vitro* that it did upon its initial isolation (without evidence of transformation). Thus, when the momentum for continued division *in vitro* is derived from incidental or purposeful immortalization of a progenitor cell, the starting cells cannot be considered to possess the stem cell property of self-renewal.

Operational stem cell definitions in vivo*: the gold standard*

In vivo operational definitions of stem cells are similar to those employed for *in vitro* studies: single cells must demonstrate proliferation and long-term self-maintenance as well as multipotentiality. *In vivo* cell proliferation in the adult brain is sometimes erroneously equated with the presence of a resident stem cell population. Conversely, stem cell populations that are quiescent *in vivo* can be missed when too much emphasis is placed on the demonstration that the cells are proliferating *in vivo*, as in the case of retinal stem cells [28]. Long-term self-maintenance *in vivo* can be demonstrated by attempting to isolate the same cell population from adult and senescent animals to determine whether it has been 'maintained' *in vivo*. It is technically difficult to label single proliferating cells *in vivo* and follow them over long periods of time to determine whether they exhibit self-maintenance and multipotentiality, but this would be the most convincing evidence. The current absence of sensitive and specific neural stem cell markers contributes to the difficulties in assessing these features *in vivo*.

Finally, what about situations in which some or all of these criteria can be met *in vitro* but not *in vivo*, or vice versa? This is a problem that is not limited specifically to neural stem cells; indeed, there are no examples of cells from any tissue system that have been demonstrated to exhibit all of the aforementioned criteria both *in vitro* and *in vivo*. Nevertheless, the validation of *in vitro* stem cell assays ultimately depends on their congruence with the stem cell as defined *in vivo*.

Despite these issues, we maintain that it is crucial to classify and study neural stem and progenitor cells separately whenever possible. This increases the predictive

power of experimental manipulations and increases the understanding of mechanisms of tissue development, growth and repair. It is equally important to understand the inherent biological differences between stem and progenitor cells, to determine which cell type is most suitable for the development of potential therapeutic applications.

Concluding remarks

The concept of a stem cell is itself in a state of flux, in part owing to recent remarkable examples of plasticity [5–9] that suggest apparently lineage-restricted stem cells have the capacity to give rise to differentiated stem cells of developmentally unrelated tissues. The rigorous definition of stem cells appears to ignore this novel property. However, this definition (unlimited self-renewal and within-tissue multipotentiality) does not necessarily exclude cross-tissue plasticity. For example, a pluripotent mesenchymal stem cell has been described recently that can contribute to most somatic tissue types when injected into an early blastocyst, but can maintain its stem cell characteristics *in vitro* and *in vivo* [41]. Nevertheless, most examples of plasticity seem to involve very rare cells and, indeed, cross-tissue plasticity might not be a property of all of the few stem cells present within any given tissue. Furthermore, given that some of the observed tissue plasticity of neural stem cells contributing to blood lineages [5] and vice versa [6,8] have not been replicated [37,42,43], it seems precarious to rebuild the concept of a stem cell on such a potentially unstable foundation.

It is undeniable that the concept, and thus the operational definition, of a stem cell is 'evolving' [4], and it is clear that the present definitions have limitations. However, this does not excuse the abandonment of the rigorous definitions that have provided useful guidance for investigation and interpretation of functional differences between distinct cell types. These definitions have proven their utility: valuable insights into neural progenitor and stem cell biology have been gained by investigating, comparing and even emphasizing the functional differences between cells.

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