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Strengths and Limitations of the Neurosphere Culture System

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Abstract

After the initial reports of free-floating cultures of neural stem cells termed neurospheres *(1,2)*, a wide array of studies using this promising culture system emerged. In theory, this was a near-perfect system for large-scale production of neural cells for use in cell replacement therapies and to assay for and characterize neural stem cells. More than a decade later, after rigorous scrutiny and ample experimental testing of the neurosphere culture system, it has become apparent that the culture system suffers from several disadvantages, and its usefulness is limited for several applications. Nevertheless, the bulk of high-quality research produced over the last decade has also shown that under the right circumstances and for the appropriate purposes, neurospheres hold up to their initial promise.

This article discusses the pros and cons of the neurosphere culture system regarding its three major applications: as an assay for neural stem cells, as a model system for neurogenesis and neural development, and for expansion of neural stem cells for transplantation purposes.

Index Entries: Neurosphere; in vitro; neural stem cells; regional specification; transplantation; clonal analysis; neuron; glia.

The Neurosphere Culture System

Various methods with slight variations in protocol exist for establishing and expanding neurosphere cultures. Generally, neurospheres are derived from a single-cell suspension of neural stem and progenitor cells isolated from the adult or fetal central nervous system (CNS), but neurosphere cultures can also be established from ES cells *(3)*. The starting population of cells is usually plated as a single-cell suspension on uncoated plastic in a N2 and/or

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B27 supplemented medium containing fibroblast growth factor (bFGF) and/or epidermal growth factor (EGF) *(2,4–7)*.

One of the major problems with the neurosphere culture system is that it appears sensitive to the culturing method used. Variations in cell density alter the microenvironment, which in turn may affect both the proliferation capacity *(6)* and the positional cues that the cells are exposed to *(8)*. Different constituents or concentrations of factors in the media *(9–11)*, method and frequency of passaging *(10)*, whether the neurosphere is dissociated before differentiation *(10)*, and the number of passages after isolation *(12)* also lead to differences in both the composition of cell types as well as the properties of the cells within each neurosphere. Because of the sensitivity for such variation in culture method, it is often hard to consolidate data from different groups *(8,13)* or even to interpret the results within the same study *(14)* to make comprehensive conclusions about the properties of the cells within the neurospheres.

Another difficulty with the system comes from the inherent properties of suspension cultures: the neurospheres represent "little black boxes" that you cannot peer into, making it hard to carefully monitor the properties of individual cells during the culture period. The neurogenic capacity of the neurosphere-expanded cells declines after extended numbers of passages *(15)*. It is not clear whether this is because progenitors with capacity to form neurons are outnumbered by glial progenitors and thus progressively lost or whether the intrinsic properties of the cells within the neurospheres change their properties over time.

Furthermore, the neurospheres are heterogeneous in nature, and only a small percentage of cells within each sphere holds the neurosphere-forming capacity *(2)*; even fewer fulfill the criteria of being neural stem cells *(16)*. Each neurosphere contains cells at various stages of differentiation (Fig. 1), including stem cells as well as proliferating neural progenitor cells and postmitotic neurons and glia *(17,18)*. This heterogeneity increases with sphere size because more differentiated cell types arise after longer time in culture. To monitor the presence of neural stem and progenitor cells in the neurospheres, we generated neurospheres from mice that expressed green fluorescent protein (GFP) from the *Sox1* locus *(19)*, enabling identification of neural stem and progenitor cells by their GFP expression *(20)*. We found that even small neurospheres contain a mix of GFP-positive and -negative cells shortly after passage (M.P., unpublished observation). Therefore, even at a very early stage, the neurospheres appear heterogeneous. This heterogeneity makes it difficult to study any specific event or cell in isolation, and studies using neurospheres should be seen and interpreted as studies on a mixed population of precursor cells and not as studies of neural stem cells.

The Neural Stem Cell Assay

The neurosphere culture system was the first in vitro system to unequivocally demonstrate the presence of cells in the adult brain with characteristics of true neural stem cells *(1,2)* and remains an extremely useful tool to analyze proliferation, self-renewal capacity, and multipotency of neural stem and progenitor cells. Testing for neurosphere-forming capacity over serial clonal passaging (Fig. 2A) followed by in vitro differentiation to show multipotency of individual spheres (Fig. 2B,C) is widely used and, when performed properly, provides the best functional assay for neural stem cells available *(4,6,21,22)*. The system is particularly attractive because clonal analysis can be performed with ease compared to adherent culture systems where, in the past, complicated labeling methods had to be employed to determine clonal relation between a cell and its progeny *(23)*. Recently, a new adherent monolayer culture system has been reported that allows for serial clonal expansion of neural stem cells *(24)*, and this culture provides a relatively uncomplicated alternative assay for neural stem cells.

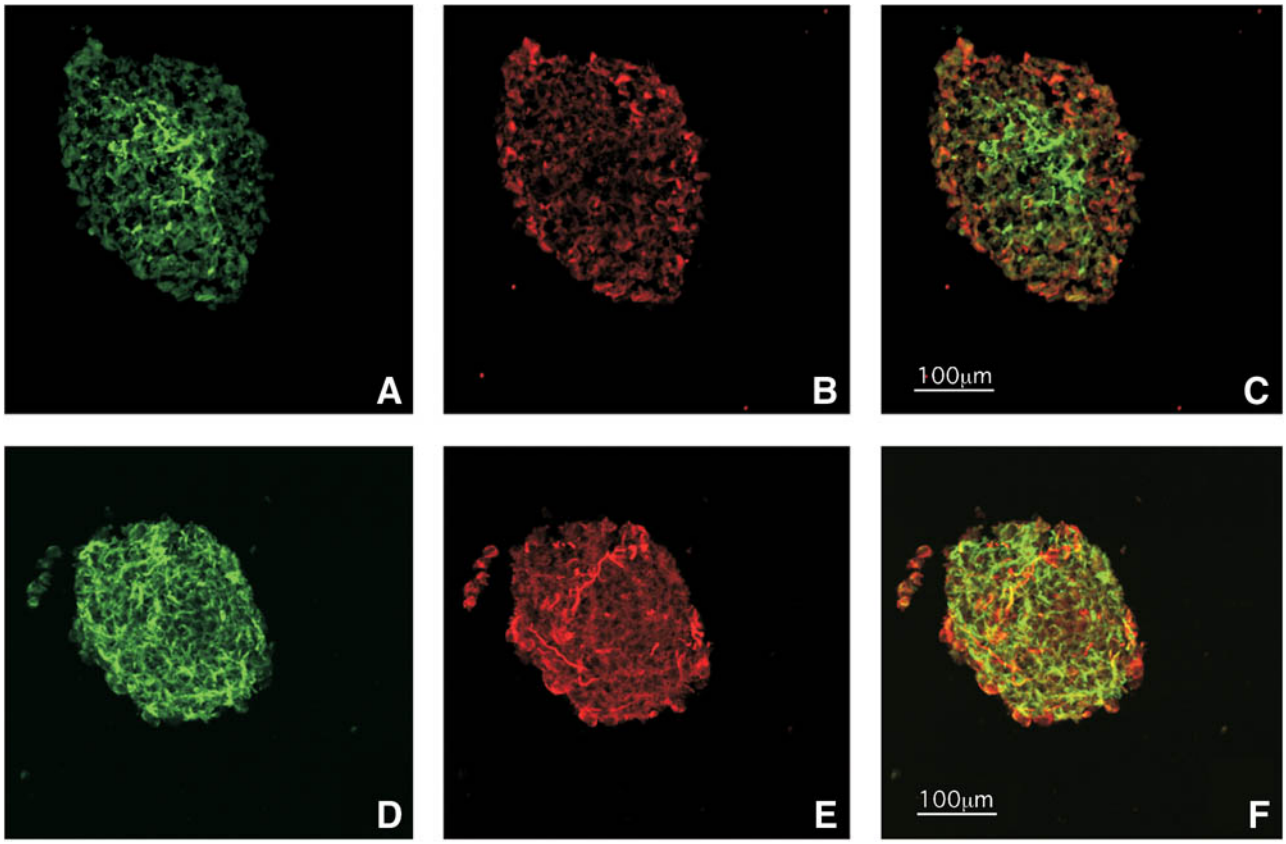

Fig. 1. Neurospheres are heterogeneous in nature with RC2 **(B,C)** and nestin-positive **(D,F)** progenitor cells generally located toward the outside of the sphere and GFAP-positive **(A,C)** cells in the center of the spheres. Few βIII-tubulin-positive neurons **(E,F)** can also be found evenly distributed within some spheres. (Figure modified from ref. *17*.)

Neurospheres as a Physiologically Relevant Model System

In addition to its function as a neural stem cell assay, the neurosphere culture system is a valuable in vitro model system to study neurogenesis and neural development. Most studies on the genetic and molecular control of regional specification of neural precursors have been performed in vivo. Complimentary in vitro approaches are useful to determine the degree of intrinsic specification present in neural precursors at various developmental time-points, as well as to study the full potential of the cells when removed from extrinsic cues provided by their normal environment. The neurospheres are a good system for such studies because they are maintained under defined serum-free conditions where the environmental cues are limited to those of surrounding cells. Additionally, it is easy to manipulate the extrinsic cues the cells are exposed to during their development by changing the environment during either the expansion or differentiation phase. This can be done by simply adding precise and variable amounts of factors of interest to the media *(25–27)* or by culturing the neurospheres together with other types of cells, such as primary cells from the developing CNS *(26,28,29)*.

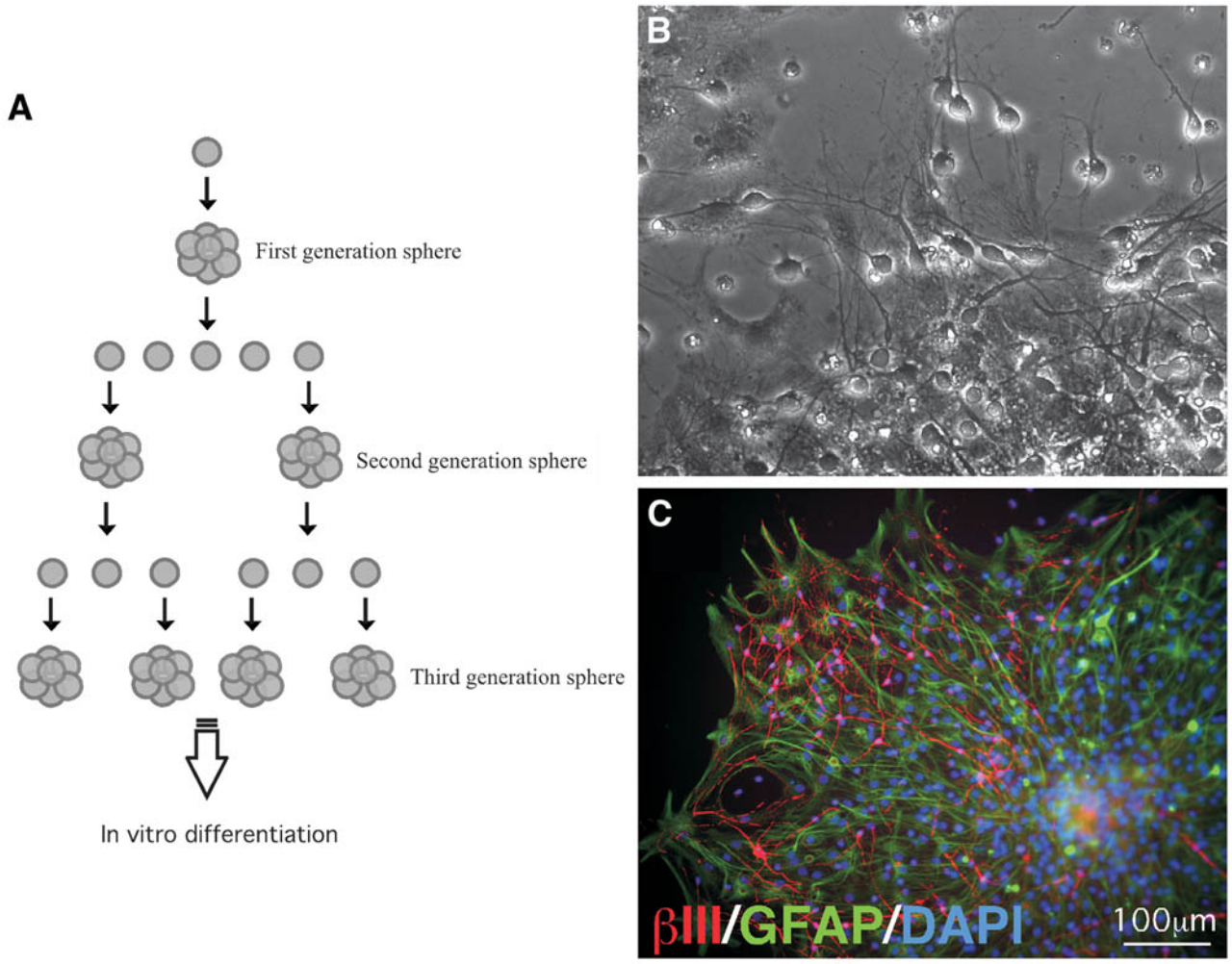

Fig. 2. The neural stem cell assay test for neurosphere-forming capacity over serial clonal passaging followed by in vitro differentiation of individual spheres **(A)**. A requirement for stem cellness is that a sphere derived from a single cell upon differentiation can give rise to all three major cell types of the central nervous system: neurons, oligodendrocytes, and astrocytes. During differentiation, cells migrate from the core of the neurospheres (bright field, **B**) and express genes such as GFAP (green, **C**) or βIII-tubulin (red, **C**), markers characteristic for astrocytes and neurons, respectively. (Color version of this figure appears online.)

Another advantage is that the intrinsic properties of the neurosphere-expanded cells can be easily and efficiently modulated by viral transduction using retroviral vectors *(8,30,31)*.

Comparative studies of the fetal brain and neurosphere cultures have shown that neurosphere-expanded neural stem and progenitor cells behave like their temporal and spatial in vivo correlates in several respects: the cells proliferative capacity and differentiation potential after exposures to different external factors varies in a manner that is reflective of the developmental stage of the donor *(6,11)*. Furthermore, the expression of many developmental control genes is maintained in a regionally specific manner in the neurosphere cultures after several passages, and the neurosphere-derived cells maintain the potential

to differentiate into neuronal subtypes characteristic of their region of origin, thereby reflecting the normal cell fate of the neural progenitors from this region *(13,28,29,32,33)*. Although this sounds promising for the use of neurospheres to model biological processes, one must use the system with caution. Notably, these studies showed only partial maintenance of regionally specific expressed genes in the neurosphere cultures. Other studies based on cells derived from the same regions of the developing brain did not detect preservation of regionally specific expression patterns *(8,27)*. These contradicting results are not always easily explained, but they highlight the concept that only minor variations in the culturing procedure may have a significant impact on the results.

Furthermore, the characteristics and behavior of stem and progenitor cells varies among regions within the CNS, and the usefulness of the neurosphere culture system as a model for studying development depends largely on the region of interest. For example, studies have shown that olfactory bulb interneurons are generated under standard differentiation conditions from neurosphere-expanded cells isolated from the lateral ganglionic eminences *(17,34)*, which give rise to olfactory bulb interneurons and striatal projection neurons during development *(35–37)*. However, striatal projection neurons are only formed from these cultures when differentiated in cocultures with primary cells from the lateral ganglionic eminences *(26)*. Similarly, all subtypes of neurons present in the cerebellum (including the granule neurons) are generated from neurosphere-expanded cerebellar cells when differentiated in cocultures with primary cerebellar cells *(29)*. Therefore, the neurosphere system is suitable to study aspects of forebrain and cerebellar development. On the other hand, successful generation of a substantial number of midbrain dopaminergic neurons from multiple-passaged neurospheres have yet to be reported, and it is not clear whether mesencephalic dopaminergic neuron precursors can be expanded or even maintained in neurosphere cultures *(38)*. Therefore, the neurosphere culture system might not be appropriate to use when studying stem and progenitor cells from certain regions of the CNS, such as the ventral midbrain.

One advantage of the neurosphere culture system can also be a drawback—namely, it allows us to study the full potential of the cells when taken out of their normal environment. Therefore, a cell that can produce all three major cell types of the CNS in vitro may not necessarily give rise to these cell types during normal development. The discrepancies between in vivo and in vitro potentials can arise because of either the lack of cell–cell interactions or the lack and/or presence of factors in the culture system. An example of this is the conversion of the bipotential dorsal spinal cord progenitor cells into a tripotential neural stem cell by the presence of bFGF *(39)*. Therefore, it is very important to be cautious in the interpretation of results and to distinguish between the potential of a cell and its actual behavior during normal development.

Limited Use of Neurospheres for Cell Replacement Therapies

Fetal mesencephalic tissue has been used as a source of dopaminergic neurons for transplantation in clinical trails of patients with Parkinson's disease *(40,41)* and in animal models of Parkinson's disease *(42,43)*. Other transplantation studies with fetal tissue from different regions of the developing brain have also shown region-specific neuronal differentiation after transplantation into the developing, neonatal, or adult rodent brain *(44,45)*. Therefore, neural progenitors isolated from the developing brain harbor a promising potential for use in cell replacement therapies but suffer from limited availability and ethical concerns. The neurosphere culture system has the potential to provide an unlimited number of neural stem and progenitor cells initially derived from either the fetal or adult CNS, thus enabling the production of an on-demand

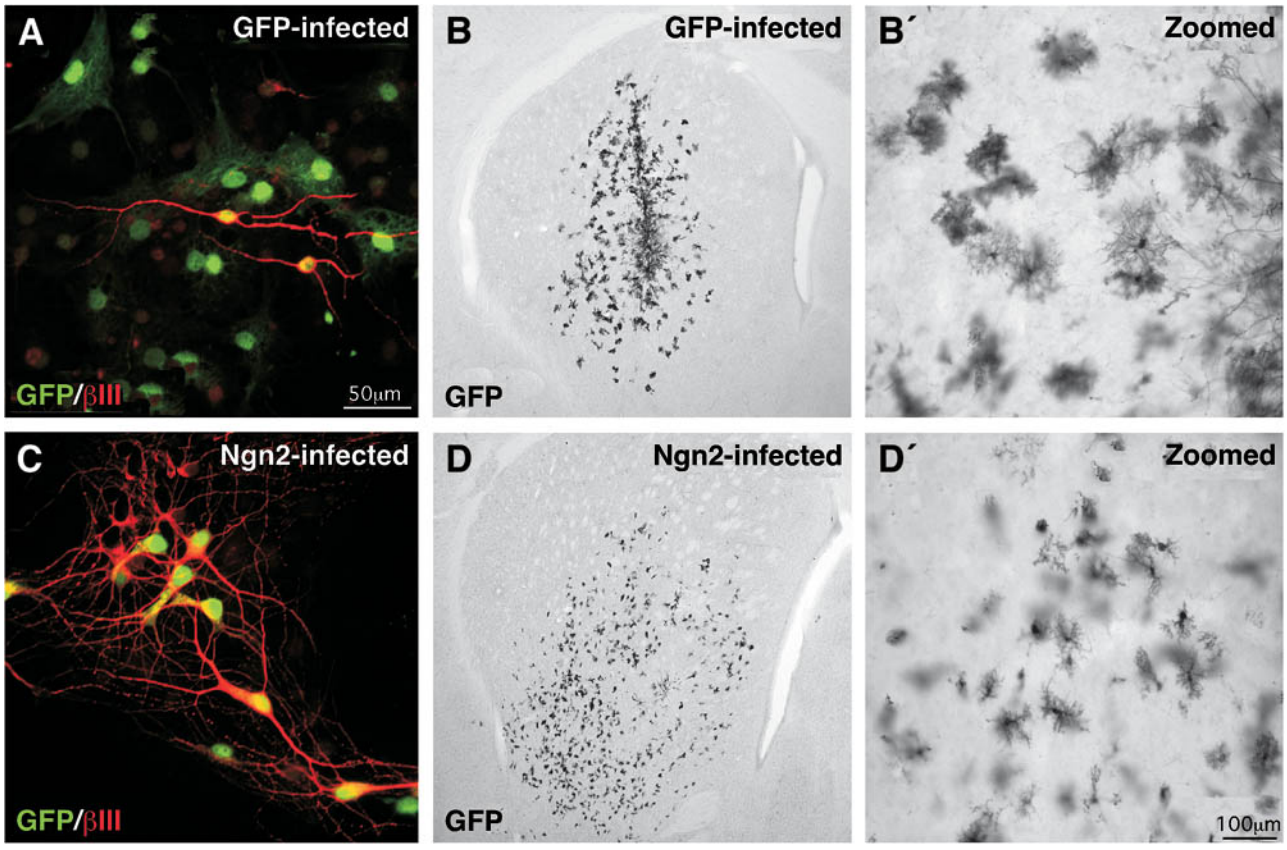

Fig. 3. Neurosphere-expanded neural stem and progenitor cells from the embryonic forebrain transfected with Ngn2-expressing retrovirus **(C)** give rise to a high number of neurons after in vitro differentiation compared to GFP-control transfected cells **(A)**. However, after transplantation into the neonatal striatum, the Ngn2-transfected cells **(D,D´)** as the GFP-control-transfected once **(B,B´)** mainly differentiate into cells with glial morphologies. The infected cells are identified by expression of GFP (green in **A**,**C**, black in **B**,**D**), whereas neurons are visualized by the expression of βIII-tubulin (red in **A**,**C**).

source of cells that can be standardized, quality tested, and relatively easily genetically modified if needed.

Although some researchers have been successful in obtaining a considerable number of neurons after transplantation of neurosphere-expanded cells into neurogenic areas of the brain *(15,46,47)*, only poor yields of neurons have been achieved when cells are engrafted into non-neurogenic areas such as the striatum *(14,48–50)*. The engrafted cells survive but differentiate into glial cells, rather than neurons. From in vitro studies, we know that the environment can have a strong influence on the differentiation of the neurosphere-expanded cells *(26,28,29)*. Therefore, the gliogenic environment of the adult and neonatal brain is likely to have a negative effect on the neuronal differentiation of the neurospheres, which can explain the poor neuronal yield after transplantation compared to after in vitro differentiation. Thus, the neurosphere-expanded cells appear to differ from the original source of cells in their susceptibility to environmental cues, as unexpanded fetal tissue generates a large number of region-specific neurons after

grafting. To abolish the negative influence of the host environment on neuronal differentiation, attempts have been made to change the intrinsic properties of the neurosphere-expanded cells by genetic manipulation. Neurosphere cultures derived from the adult brain that ectopically express the proneural gene Neurogenin2 (Ngn2) have an increased potential for generation of neurons in vitro *(30)*. This is also the case when neurospheres generated from the embryonic brain are engineered to express Ngn2 (authors' unpublished observation). Ngn2-expressing neurospheres generate neurons at a much higher frequency (90%) than control-infected cultures (5%) when differentiated in vitro (Fig. 3A,C). However, the same cell cultures do not give rise to a substantial number of neurons after transplantation (unpublished data; Fig. 3B,B′,D,D′). This discrepancy between in vitro and in vivo differentiation has been observed elsewhere *(51)*, suggesting that neurosphere-expanded cells are not easily committed to a neuronal fate and that expression of one gene normally involved in neuronal commitment is not sufficient to promote neuronal differentiation in a complex environment.

Conclusion

The neurosphere culture system has been shown to be useful for biological studies of developmental processes *(29,52)*; in many cases, it is crucial as a functional assay to test for neural stem cells characteristics. However, because of its heterogeneous character and the apparent sensitivity to variations in the methodological procedure, it has to be used with great caution when studying biological processes, and it is sometimes difficult to merge findings from different labs to gain a more complete understanding of a particular process.

This, in combination with the progressive loss of neurogenic potential with passaging and poor yield of neurons after transplantation of neurosphere-expanded cells into the nervous system, has raised a need for development of a new culturing system that supports the expansion of neural stem and progenitor cells. To have major advantages over the neurosphere culture system, the new system needs to be robust, homogeneous, stable over time, and to continuously give rise to a large percentage of neurons in vitro as well as in vivo.

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