

## Neurosphere Assays: Growth Factors and Hormone Differences in Tumor and Nontumor Studies

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### ABSTRACT

The “no new neuron” dogma that the brain is quiescent throughout adult life has been challenged by the discovery of cells with stem cell-like qualities of self-renewal and multipotency in the subventricular zone of the lateral ventricles and the dentate gyrus of the hippocampus in adults. This self-renewing capacity also makes these neural stem cells a possible source of brain tumors, which was supported by the discovery of self-sustaining brain tumor stem-like cells in cancers such as glioblastoma multiforme. Neurosphere assays are the standard for studying these stem-like cells in both normal and cancer tissues. Despite the importance of these assays, there is no stan-

dardized protocol to allow for a comparison of results because several studies use different growth factors and hormones at different concentrations. The primary objective of this study is to review the literature for both nontumor and tumor studies to assess their respective neurosphere assay components. We found significant variation in assay components, namely hormones and growth factors, as well as their respective concentrations. This illustrates the need for a standardized protocol to allow proper comparison among studies and a better assessment of the effects of different factors. *STEM CELLS* 2006;24:2851–2857

### INTRODUCTION

The dogmatic view of the brain as immutable and incapable of neurogenesis was first challenged by Altman in the 1960s, [1] and continued with Goldman and Nottebohm in the 1980s [2]. Altman identified the presence of new neurons within restricted areas of the rat brain by using [<sup>3</sup>H]thymidine-labeled cells and light microscopy [3]. Goldman and Nottebohm further demonstrated telencephalic neurogenesis in female canaries [2]. Since then, several studies have shown that a discrete population of cells within the subventricular zone (SVZ) of the forebrain and the dentate gyrus of the hippocampus, neural stem cells (NSCs), are capable of generating mature cells throughout adult life [4–7].

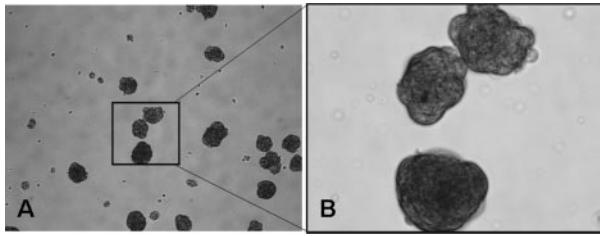
This has led to the discovery of a cell population within brain tumors that displays many key features of NSCs, [8–11] including continuous self-renewal [12], extensive migration [13], potential for differentiation [12, 13], and many of the same cell surface markers [8, 9]. The characteristics of these brain tumor stem cells (BTSCs) may be responsible for the growth and recurrence of certain tumors, as well as their resistance to therapeutic regimens [14]. Therefore, BTSCs may represent a new target for cancer therapy.

Advances in stem cell research have created a need for novel experimental techniques to study these cells. Currently, neurosphere assays remain the standard for identifying stem cell populations, allowing for isolation and characterization of NSCs (Fig. 1) and, most recently, BTSCs [14, 15]. These assays consist of a selective serum-free culture system that allows NSCs and BTSCs to proliferate and generate multipotent clones, or neurospheres, while the more differentiated cells die out [15]. The components of these culture systems, however, are not uniform and vary significantly between studies. This review provides a comprehensive summary of the different neurosphere culture media that have been used to study NSCs and BTSCs, and describes their various components.

### MATERIALS AND METHODS

The PubMed database current to May 2006 was filtered using the search terms “neurosphere,” “assay,” “neural stem cells,” or “brain tumor stem cells,” and limited to English-language papers. Eleven non-brain tumor and nine brain tumor neurosphere assay studies were selected for inclusion into the review for comparison. Studies were excluded if they cited another study’s protocol or did not clearly state the components of their neuro-

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**Figure 1.** Neurospheres derived from human subventricular zone. **(A):** Low-magnification image from a light microscope demonstrating neurospheres created from differentiated human subventricular zone astrocytes. **(B):** Higher magnification image of some of the neurospheres in **(A)**. The neurosphere assay used to create these neurospheres, consisted of Dulbecco's modified Eagle's medium/F-12 with fibroblast growth factor (20 ng/ml) and epidermal growth factor (10 ng/ml), as well as insulin, progesterone, transferrin, selenium, putrescine, and glutamine.

sphere media. The culture media and cell types were obtained from the methods section of each paper and tabulated (Tables 1 and 2) for comparison.

## RESULTS

### Nontumor Neurosphere Studies

Eleven studies were obtained with non-brain tumor neurosphere assays from 1992 to 2006 (Table 1) [7, 8, 16–24]. Seven of the studies were derived from rodent brains (six mice [16–18, 21–23], one rat [20]), and four of the studies were from human brains (one fetal tissue [19], two subependymal zone/SVZ [7, 8], one SEZ and hippocampus [24]). Nine of these studies used a serum-free medium, which was Dulbecco's modified Eagle's medium (DMEM) supplemented with F-12 [7, 18, 19, 21] or B27 [19], methylcellulose-based medium [8], or an unknown serum-free medium [16, 17, 22, 23]. Two studies, however, used bovine serum albumin (BSA) [20, 24].

The components that were added to the media varied from study to study. The use of growth factors was not uniform. Five of the studies used both epidermal growth factor (EGF) and fibroblast growth factor (FGF) at varying concentrations between 10 and 20 ng/ml [7, 8, 19, 22, 23], whereas the remaining studies used only FGF [17, 20] or EGF [16, 18, 21]. One of the studies used EGF, FGF, and platelet-derived growth factor (PDGF) [22]. Likewise, the use of hormones was not consistent. Four of the studies used the hormones progesterone and insulin as part of their neurosphere assay [18, 20, 21, 23]. Other components that varied between studies included antibiotics (two studies [19, 20]), antimycotics (one [19]), transferrin (three [18, 20, 23]), heparin (two [19, 23]), and selenide (three [18, 20, 23]).

### Tumor Neurosphere Studies

Nine studies were obtained with brain tumor neurosphere assays from 1999 to 2006 (Table 2). [8–10, 12, 13, 25–28] Eight of these studies involved human brain tumors, including glioblastoma multiforme (GBM), grade II and III astrocytomas, medulloblastoma, ependymomas, and gangliomas. [8–10, 12, 13, 26–28] The remaining study was obtained from a C6 rodent glioma cell line [25]. The media varied in each of these studies. Seven of the studies used serum-free media, which included DMEM supplemented with F-12 [13], neurobasal medium supplemented

with N2 [12, 26, 28], methylcellulose-based medium [8], and an unknown other serum-free medium [9, 10, 27]. Two of the studies used medium supplemented with BSA [25, 26].

As with nontumor neurosphere assays, the components added to brain tumor neurosphere media were not uniform across the studies. The studies used different types and concentrations of growth factors. Seven studies used both EGF and FGF at concentrations between 10 and 50 ng/ml [8–10, 12, 13, 26–28], whereas two studies used FGF [25, 28]. One study used PDGF [25], three studies used leukemia inhibiting factor (LIF) [9, 10, 27], and two studies used neural survival factor (NSF) [9, 28]. With regard to hormones, three studies [12, 25, 26] used progesterone and insulin, whereas the remaining five studies [8–10, 13, 27] did not add any hormones. The other components that varied among studies included antibiotics (one study [25]), transferrin (three studies [12, 25, 26]), selenide (three studies [12, 25, 26]), and putrescine (three studies [12, 25, 26]).

## DISCUSSION

The no new neuron doctrine that the brain is quiescent throughout adult life and devoid of neural stem cells has dominated neuroscience thinking for centuries. This began to change with Altman's discovery of new neurons in rats in 1966, [3], and the idea was further questioned with Goldman and Nottebohm's finding of neurogenesis in canaries in 1983 [2]. Studies since then have localized neurogenesis to the SVZ of the lateral ventricles and the dentate gyrus of the hippocampus [4–6]. These regions consist of mitotically active neural stem cells and progenitor cells capable of differentiating into astrocytes, neurons, and oligodendrocytes [17]. This self-renewing capacity also makes neural stem cells a possible source of brain tumors, a concept validated by the discovery of self-sustaining brain tumor stem cells in human GBM by Ignatova et al. in 2002 [8]. This has led to an era in neuroscience research in which neural stem cells are being studied actively for both their regenerative capacity and their devastating neoplastic potential.

Neurosphere assays were initially used by Reynolds and Weiss in 1992 to isolate neurospheres from the mouse striatum [29], and remain the standard for determining the presence of these stem-like cells in both tumor and nontumor tissue because they can determine whether a particular cell has self-renewing and multipotential capabilities [14, 15]. This assay creates conditions in which stem-like cells are able to continually divide and form multipotent, undifferentiated clones called neurospheres in vitro [10, 15, 27, 30, 31]. These conditions usually include cells being grown in serum-free medium and on non-adherent plates because unknown serum factors and adherence both promote differentiation [14, 29]. In addition, mitogens including EGF [18, 23, 29] and/or FGF [17, 23] are also used frequently because they facilitate neural stem cell proliferation. The neurospheres that form from these conditions can be serially dissociated and replated to generate additional neurospheres. Multipotent clonal cells within the neurospheres can further form neurons, astrocytes, and/or oligodendrocytes. This has been demonstrated with the identification of markers specific for neurons (TuJ1), astrocytes (glial fibrillary acidic protein), and oligodendrocytes (Oli4) [17]. In contrast, non-stem cells lack these features of self-renewal and multipotency and are theoretically eliminated with serial passages.

**Table 1.** Studies with non-brain tumor neurosphere assays

Paper	Senior authors	Neurosphere media				Cell type
		Media	Growth factors	Hormones	Other	
Reynolds et al. [16, 29]	Reynolds and Weiss	Serum-free media	EGF (20 ng/ml)	None	None	Adult mouse striatum
Gritti et al. [17]	Vescovi	Culture media	bFGF (20 ng/ml)	None	None	Adult mouse striatum
Reynolds and Weiss [18]	Reynolds and Weiss	DMEM/F-12 + 5 mM HEPES	EGF (varying conc.)	Insulin (25 $\mu$ g/ml), progesterone (20 nM)	Glucose (0.6%), sodium bicarbonate (3 mM), glutamine (2 mM), transferrin (100 $\mu$ g/ml), putrescine (60 $\mu$ M), sodium selenite (30 nM)	Adult mouse striatum
Svendsen et al. [19]	Caldwell	DMEM/F-12 + B27	EGF (20 ng/ml), FGF2 (20 ng/ml)	None	Penicillin G, streptomycin sulfate, amphotericin B (1:100), heparin (5 $\mu$ g/ml)	Human fetal tissue
Doetsch et al. [21]	Alvarez-Buylla	DMEM/F-12 + N2, HEPES	EGF (20 ng/ml)	Insulin (0.02 mg/ml), N2 (insulin, progesterone, putrescine, selenide)	Glucose (0.6%), L-glutamine (2 mM)	Adult mouse subventricular zone
Gritti et al. [23]	Vescovi	Control medium	EGF (20 ng/ml), FGF2 (20 ng/ml)	Insulin (0.025 mg/ml), progesterone (6.3 ng/ml)	Glucose (0.6%), L-glutamine (2 mM), putrescine (9.6 gm/ml), sodium selenite (5.2 ng/ml), transferrin (0.1 mg/ml), heparin (2 $\mu$ g/ml)	Adult mouse striatum
Kukekov et al. [24]	Steindler	N2 medium + BSA (5%)	None	None	None	Human SEZ and hippocampus
Ignatova et al. [8]	Steindler	0.8% Methyl-cellulose-based medium	EGF (10 ng/ml), FGF2 (10 ng/ml)	None	None	Human SEZ
Ben-Hur et al. [20]	Abramsky	DMEM/F-12 + BSA (0.05%)	FGF2 (10 ng/ml)	Insulin (25 $\mu$ g/ml), progesterone (20 nM)	Gentamycin (25 $\mu$ g/ml), sodium selenite (30 nM), putrescine (60 $\mu$ M), glutamine (2 mM), apo-transferrin (10 mg), sodium-pyruvate (1 mM), D-biotin	Newborn rat striatum
Sanai et al. [7]	Alvarez-Buylla	DMEM/F-12	FGF2 (20 ng/ml), EGF (20 ng/ml)	None	None	Human SVZ
Einstein et al. [22]	Ben-Hur	Serum-free medium	bFGF (10 ng/ml), EGF (20 ng/ml), platelet-derived growth factor-AA (10 ng/ml)	None	None	Newborn mouse striatum and cerebral hemisphere

Abbreviations: BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle's medium; EGF, epidermal growth factor; FGF, fibroblast growth factor; SVZ, subventricular zone.

**Table 2.** Studies with tumor stem cells and their respective culture media

Paper	Senior authors	Neurosphere media				Cell type
		Media	Growth factors	Hormones	Other	
Ignatova et al. [8]	Steindler	0.8% Methylcellulose-based medium	EGF (10 ng/ml), FGF2 (10 ng/ml)	None	None	Human anaplastic astrocytoma and recurrent GBM
Singh et al. [9]	Dirks	Serum-free medium	EGF (20 ng/ml), bFGF (20 ng/ml), LIF (10 ng/ml), NSF (1×)	None	<i>N</i> -Acetylcysteine (60 μg/ml)	Human medulloblastoma, pilocytic astrocytoma, grade 2 astrocytoma, ependymoma, ganglioglioma
Hemmati et al. [10]	Kornblum	Growth medium	EGF (20 ng/ml), bFGF (20 ng/ml), LIF (20 ng/ml)	None	None	Human pediatric anaplastic astrocytoma, GBM, medulloblastoma, desmoplastic medulloblastoma
Galli et al. [13]	Vescovi	DMEM/F-12	EGF (20 ng/ml), FGF2 (20 ng/ml)	None	None	Human GBM
Kondo et al. [25]	Taga	DMEM + BSA (100 μg/ml)	bFGF (10 ng/ml), PDGF (10 ng/ml)	Insulin (10 μg/ml), progesterone (60 ng/ml)	Penicillin (50 U/ml), streptomycin (50 μg/ml), transferrin (100 μg/ml), putrescine (16 μg/ml), sodium selenite (40 ng/ml), <i>N</i> -acetylcysteine (63 μg/ml), forskolin (5 μM)	Rat gliomas C6 cell line
Yuan et al. [27]	Yu	Serum-free medium	EGF (20 ng/ml), bFGF (20 ng/ml), LIF (20 ng/ml)	None	None	Human GBM
Taylor et al. [26]	Gilberston	Neurobasal medium + N2, B27, BSA (50 μg/ml)	EGF (20 ng/ml), bFGF (20 ng/ml)	Insulin, progesterone	L-Glutamine (2 mM), transferrin, putrescine, selenide	Human ependymoma
Lee et al. [12]	Fine	Neurobasal medium + N2 (0.5×)	EGF (50 ng/ml), bFGF (50 ng/ml)	Insulin, progesterone	Transferrin, putrescine, selenide	Human GBM
Phillips et al. [28]	Aldape	Neurobasal medium + N2	EGF (20 ng/ml), bFGF (20 ng/ml), NSF	None	None	Human GBM, grade III gliomas

Abbreviations: BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle's medium; EGF, epidermal growth factor; FGF, fibroblast growth factor; GBM, glioblastoma multiforme; LIF, leukemia inhibiting factor; NSF, neural survival factor; PDGF, platelet-derived growth factor.

Neurosphere assays, despite their utility, also have some limitations. First, neurosphere assays are an *in vitro* phenomenon and do not occur *in vivo* [10, 15, 27, 30, 31]. This phe-

nomenon could theoretically reflect a peculiarity of the assay itself and result from manipulation of the experimental environment that does not naturally occur *in vivo* [15]. Second, neuro-

spheres are thought to arise from clonal divisions of a single stem-like cell with self-renewal and multipotential capabilities. However, non-stem cells can also produce secondary or even tertiary neurospheres [32] but, unlike true neurospheres, cannot continue to form neurospheres with continued passaging. Furthermore, cell-to-cell fusions that occasionally occur between neural stem cells and/or progenitor cells can confound the assessment of multipotency, as well as molecular and genetic studies [33]. Finally, neurosphere assays do not provide an accurate calculation of stem cell frequency because the majority of cells in the neurospheres (approximately 97.6% [15]) are not viable and may not form neurospheres [15].

Despite the importance of neurosphere assays, there is no standardized protocol to allow for a comparison of results. Reynolds' and Weiss' original studies used a serum-free medium containing 20 ng/ml of EGF [16, 29], but a review of the literature has shown significant variation in the components of each assay in both non-brain tumor and brain tumor studies since this landmark study. Vescovi [14] states that the neurosphere approach represents a serum-free culture system, but this review shows that several of the studies use serum in their media [20, 24–26]. The serum-free studies are also inconsistent because they each use different serum-free media, ranging from neurobasal medium [12, 26] to DMEM/F-12 [7, 13, 18–21] to a methylcellulose-based medium [8]. The use of mitogens is further confounding, where studies use any combination of EGF, FGF, PDGF, LIF, and even NSF. The use of hormones also varies, where some studies use progesterone and/or insulin, while other studies use neither. The other additives that are notably different include antibiotics, antimycotics, transferrin, selenide, and putrescine. This variability in media components and “micronutrients” is not limited to neurosphere assays but is also seen in sarcosphere [34, 35], mammosphere [36, 37], and leukemia stem cell assays [38, 39].

Each of these components may have different and significant effects on the creation of neurospheres and, most importantly, on the interpretation of data. NSCs and BTSCs are usually grown on serum-free medium because serum irreversibly differentiates stem cells [40, 41]. Growth factors also have an effect on these assays. EGF in mice directly increases proliferation and survival of precursor cells in the SVZ, and enhances the generation of astrocytes [42, 43]. FGF also increases neurogenesis, but additionally protects neurons against injury-

induced degeneration [44, 45]. PDGF potentiates FGF's proliferative capacity [46], and LIF is required for the long-term replication of EGF-responsive neural stem cells [47, 48]. The removal of these mitogens induces differentiation [14, 49]. Hormones, like mitogens, also affect neurospheres. Insulin and insulin-like growth factor (IGF-1) have neurotrophic properties and increase progenitor cell proliferation [50]. Progesterone interestingly decreases proliferation in the SVZ [51, 52]. Therefore, the use of mitogens and/or hormones can have adverse effects on experimental results.

A number of micronutrients are also included in some neurosphere assays. Selenium, or sodium selenite, has anticancer properties of an unknown mechanism [53]. Glutamine is an amino acid that is shuttled between astrocytes and neurons in a process that couples ammonia metabolism to the synthesis of glutamate and  $\gamma$ -aminobutyric acid (GABA) neurotransmitters [54]. An adequate supply of glutamate is necessary for glioma growth and invasion [55, 56]. Putrescine is a low molecular weight amine that provides neuroprotection by stabilizing cellular membranes and nuclear material and is necessary for growth, replication, and tumorigenesis [57]. Transferrin is a glycoprotein that provides extracellular iron storage and transport, but also acts as an extracellular antioxidant by binding to iron and preventing it from catalyzing production of free radicals [58].

## CONCLUSION

The discovery of these NSCs and BTSCs opens the door to potential treatments for degenerative diseases such as Parkinson and devastating cancers such as GBM, which has a mean lifespan of 14.6 months after diagnosis [59]. The best means of assessing these types of stem cells is through neurosphere assays. To effectively perform these studies and compare results, however, there must be a standardized protocol and/or at least a discussion related to this issue when authors compare their data to other studies to allow proper comparison among studies and potentially a better assessment of the effects of different factors. The wide variation in media components used by each group confounds data interpretation and limits effective sharing of information in the research community. Progress in this area will help us move forward in our study of novel therapies for neurological diseases.

## DISCLOSURES

The authors indicate no potential conflicts of interest.

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