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The Culture of Neural Stem Cells

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ABSTRACT

A stem cell has three important features. Firstly, the ability of self-renewal: making identical copies of itself. Secondly, multipotency, generating all the major cell lineages of the host tissue (in the case of embryonic stem cells—pluripotency). Thirdly, the ability to generate/regenerate tissues. Thus, the study of stem cells will help unravel the complexity of tissue development and organisation, and will also have important clinical applications. Neural stem cells (NSCs) are present during embryonic development and in certain regions of the adult central nervous system (CNS). Mobilizing adult NSCs to promote repair of injured or diseased CNS is a promising approach. Since NSCs may give rise to brain tumor, they represent in vitro models for anti-cancer drug screening. To facilitate the use of NSCs in clinical scenarios, we need to explore the biology of these cells in greater details. One clear goal is to be able to definitively identify and purify NSCs. The neurosphere-forming assay is robust and reflects the behavior of NSCs. Clonal analysis where single cells give rise to neurospheres need to be used to follow the self-renewal and multipotency characteristics of NSCs. Neurosphere formation in combination with other markers of NSC behavior such as active Notch signaling represents the state of the art to follow these cells. Many issues connected with NSC biology need to be explored to provide a platform for clinical applications. Important future directions that are highlighted in this review are; identification of markers for NSCs, the use of NSCs in high-throughput screens and the modelling of the central nervous development. There is no doubt that the study of NSCs is crucial if we are to tackle the diseases of the CNS such as Parkinson's and Alzheimer's. J. Cell. Biochem. 106: 1–6, 2009.

KEY WORDS: NEURAL STEM CELLS; NEURAL PROGENITORS; NEUROSPHERES; CNS INJURY AND DISEASE

Stem cells represent a unique cell-type that serves to form, maintain, and regenerate the tissues/organs they reside in. In adult, it is likely that each tissue has a pool of stem cells that are maintained under strict growth control and can be mobilized to intervene in injury scenarios [Evans and Potten, 1991]. Thus, the study of the molecular, cellular, and developmental biology of stem cells is a very powerful approach to understanding the organisation and function of complex tissues and organs, such as the brain. The possibility that neural stem cells (NSCs) could be used to regenerate the brain gives their investigation extreme importance from a clinical perspective for treatment of diseases like Parkinson's [Sanberg, 2007]. Moreover, the hypothesis that errant growth of NSCs could give rise to brain tumors [Singh et al., 2004; Sanai et al., 2005; for reviews see Fomchenko and Holland, 2006; Galderisi et al., 2006] makes it even more compelling to investigate the biology of these cells.

Our understanding of NSCs is very basic. There are many fundamental issues that need to be resolved before clinical utility can be clearly formulated. For example, what marker(s) will allow us to purify NSCs to homogeneity, what are the optimal survival, growth, and differentiation conditions for NSCs and which proteins and pathways allow NSCs to maintain their phenotype? In this review, I will discuss mouse cells derived from embryonic brain for reasons of focus and because these cells serve as an excellent model for NSCs as a whole. The ability to isolate and study cells from transgenic (e.g., promoter-GFP reporters), mutant, knockout, and disease model mice, makes these NSCs a very powerful system. I will highlight where we stand on some of these important issues of NSC biology. In a wider context, if we are to approach dissecting the complexity of the central nervous system (CNS), we have to generate new cellular models. I believe that the study of NSCs will break down some of the barriers of complexity and provide an experimentally tractable system to understand the CNS in form and function.

DEFINITION OF A STEM CELL

At the outset, it is important to define what a stem cell is. The general definition of a stem cell arises from many years of work on hematopoietic and embryonic stem cells. These stem cell types represent the best studied and understood stem cells. It should be pointed out that even in the case of the hematopoietic stem cells we still do not have markers that allow us to purify

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these cells definitively. Stem cells have the ability to do the following:

1. Proliferate and self-renew (making identical copies of themselves) for a number of generations.
2. Differentiate into the major (if not all) lineages of the tissue they are derived from. In the case of NSCs this is the characteristic of multipotency.
3. Regenerate the tissue and/or organ they were derived from.

If all these criteria can be met from a cell within a population then, it can be defined as a stem cell. Invariably, due to technical limitations, it may not be possible to show that the cells can regenerate the tissue and/or organ they were derived from. In the case of NSCs, it is impossible to assess the ability of these cells to generate the whole CNS. Thus, the approach taken by most researchers is to transplant the putative NSCs and show that in vivo the cells can survive and generate the relevant cell types.

ADULT NSCS AND NEUROGENESIS

A large number of studies have demonstrated the existence of adult NSCs and adult neurogenesis [Arlotta et al., 2003; Lie et al., 2004; for reviews see Ming and Song, 2005]. This profound observation has led to a paradigm shift in how the CNS is viewed. The presence of NSCs in adult suggests that the CNS is like other tissues and has the potential to regenerate through endogenous pathways. More recent work has focused on investigating whether endogenous NSCs can give rise to neurons that integrate and function within the CNS. Two important studies in particular provide strong support for the role of NSCs in endogenous CNS repair outside known neurogenic areas [Magavi et al., 2000; Chen et al., 2004]. It seems that the endogenous NSC pathway in adult is stimulated by insults, injury, and disease but fails to unleash a complete response. Failure of the response at the level of cell survival, differentiation, and integration may explain the problems of endogenous pathways to regenerate CNS [Arvidsson et al., 2002]. Hostile environments including proteins such as Nogo and chondroitin sulphate proteoglycan are also a significant barrier to CNS regeneration. Nevertheless, there are opportunities to promote endogenous repair by targeting these failures. For further information on the important areas of adult neurogenesis and CNS repair the reader is directed to an excellent recent review [Jagasai et al., 2006].

IN VITRO CULTURE OF NSCS

To study NSCs we have to isolate, purify, and expand these cells in vitro and take them out of their natural niche. Embryonic NSCs in vivo are undergoing complex time-dependant niche changes to allow them to fulfill their roles in the developing CNS. Adult NSCs have to be kept under strict growth control to avoid over-expansion in the mature CNS. The composition of the niche of stem cells is an important subject, which must be further explored [Potten and Loeffler, 1990]. Thus we have to identify factors released by NSCs and understand the cell contact requirements that help maintain these cells in their physiological state.

The isolation of NSCs from the CNS through the neurosphere (NS) formation assay (NFA) was first described in 1992 [Reynolds and Weiss, 1992; Reynolds et al., 1992]. The idea behind the NFA is that cells able to form NS are likely to be NSCs and this was analyzed by the ability of these cells to passage (self-renew) and differentiate into the three major CNS lineages; astrocytes, oligodendrocytes, and neurons (Fig. 1). These two criteria: self-renewal and multipotency, fulfill the central definition of a stem cell. The NFA was used to isolate cells from both embryonic and adult tissue [Reynolds and Weiss, 1992; Reynolds et al., 1992]. NS are spheroid structures that consist of cells with a rich extra-cellular matrix (ECM) surrounding. NS are enriched for β1 integrins, epidermal growth factor receptor, and cadherins [Jacques et al., 1998; Lobo et al., 2003; Campos et al., 2004]. NS produce their own ECM molecules (laminins, fibronectin, chondroitin sulphate proteoglycans) and growth factors [Lobo et al., 2003]. Initial cell–cell contacts are retained by dividing cells in suspension cultures. The 3D structure of NS creates a niche that is more physiologically relevant than 2D culture systems and allows the modeling of a dynamic changing environment such as varying growth factor or nutrient concentrations. It is clear that the NFA has been critical in investigating the presence of NSCs in both the

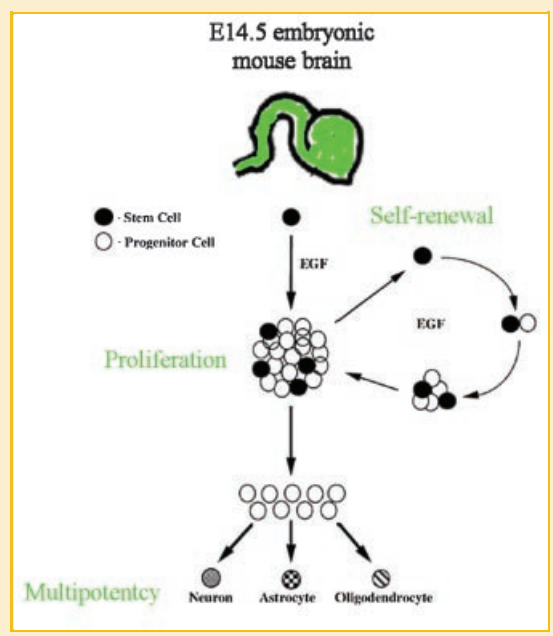

Fig. 1. Isolation of NSCs from embryonic brain. A schematic of the culture of NSCs in NS culture is shown, focusing on the steps of self-renewal; NS formation, proliferation; NS growth and differentiation; NSCs multipotency. The cortex of 14.5-day transgenic actin promoter-GFP mouse embryos is isolated and dissociated in Dubecco's modified Eagle's medium. Cells are then plated at 200,000/ml into 10 cm culture dishes. Culture medium is DMEM/F-12 including B27 supplement with EGF. Free-floating NS can be seen to form from the second day of plating. Subsequently, NS are passaged every 7 days by mechanical dissociation and replated in growth medium. NSCs start to transform after passage 10.

embryonic and adult CNS as well as regions of the CNS [Gritti et al., 1995; Weiss et al., 1996]. Important features of the NFA include; (i) the identification of NSCs, (ii) conceptual simplicity and robustness, (iii) a starting point for studies of molecular mechanisms of neurodevelopment, (iv) a means to screen for factors, chemicals, and drugs for brain cancer and CNS diseases (see future directions below).

The number of NS formed from 100 cells plated gives the NS forming units (NFUs). A critical issue here is how strict the relationship between NSCs and NFUs is. Can some NPs also form NS and how do we distinguish which cells, NSCs or NPs, are giving rise to NS. Reynolds and Rietze [2005], in their interesting perspective, suggest that growth rates of cells in NS culture can be used to test the idea of whether NFUs truly reflect the number of NSCs in any particular culture. Their starting point is to say that clonal NFU analysis suggest that there is 2.4% NSCs in NS cultures. They then derive theoretical growth rates based on this 2.4% value and compare against growth rates observed from bulk culture of NS over 10 passages. They find that bulk cultures have much lower growth rates than would be predicted from the presence of 2.4% NSCs. Based on bulk culture growth rates (and other assumptions) they estimate that there is only 0.16% NSCs within NS cultures and conclude that NFU grossly overestimate the number of NSCs, and therefore that NPs have the ability to form NS. A major problem with this analysis is that we do not know if the bulk culture conditions used are optimal for NSC growth. If survival or proliferation of NSCs is compromised in bulk culture then estimates of NSC numbers can only represent a lower limit.

In our own work on mouse NSCs, we have found that NFUs can vary over a wide range depending on growth conditions. For example, at clonal density, NFUs can drop to 0.1 or lower. Interestingly, addition of conditioned medium derived from NS growth over 1 week can stimulate both clonal and low-density NFUs by approximately eightfold (Muly Tham, Srinivas Ramasamy, Ashray Ramachandran, and Sohail Ahmed, unpublished data). These results support the view that NSCs survival is critically dependent on growth conditions. The dynamic nature of the NSC niche, which involves both temporal changes in ECM and growth factor levels, is yet to be studied. The effect of other environmental cues such as pH and oxygen tension in influencing NSCs self-renewal is unknown. Both survival capacity and the proliferation potential of NSCs are fundamental for stem cell maintenance. In particular the non-homogenous distribution of cells in bulk culture can be shown to promote the survival of cells that are near to each other and also for cells that are more robust to change in the extra cellular environment (Ashray Ramachandran and Sohail Ahmed, unpublished data).

A further complication of the analysis of Reynolds and Rietze [2005] is that in NS bulk culture at high density there is significant NS aggregation [Singec et al., 2006; Mori et al., 2007; Coles-Takabe et al., 2008]. This phenomenon has been observed most directly by differential marking of cells (with actin promoter driven EYFP and dsRed) followed by NS color analysis [Coles-Takabe et al., 2008]. The phenomenon of NS aggregation is clearly seen by the fact that large NS are seen to form in 3 days of culture of dissociated cells where only approximately three cell divisions are possible. NSCs double every 20 h approximately. At 3 days NS should only contain on average eight cells. The lack of clonality within any one sphere prevents the use of the bulk culture growth method for modeling purposes as well as multipotency assays. For the reasons outlined above the Reynolds and Rietze analysis [2005] does not undermine the relationship between NFUs and NSCs.

To date there are no definitive markers for NSCs. Therefore, we have to follow cell behavior as an indicator for NSCs. The ability to form NS is still a good indicator of the presence of NSCs. In light of the lack clonality of bulk cultures we have to use low-density cultures or immobilized NS [Singec et al., 2006; Mori et al., 2007; Coles-Takabe et al., 2008; Louis et al., 2008] to determine self-renewal and multipotency. Recently, Louis et al., [2008] have suggested that at least two discreet size populations of NS form after 21 days of culture in collagen gels (immobilized NS), and that the big NS passage (self-renew), while the small NS do not. Louis et al. [2008] interpret this to mean that big NS are derived from NSCs while small NS are not. Thus the ''big NS assay'' could also be a behavioral indicator of the presence of NSCs. However, it will be important to characterize the small and big NS further to see if they truly represent distinct cell populations. Lastly, a recent analysis places enhanced Notch signaling at the doorstep of NSCs behavior [Mizutani et al., 2007]. Their central finding is that Notch effector C-promoter binding factor 1 (CBF1) activity followed by promoter GFP expression yields two cell populations from NS culture, a high and a low population. The CBF1 high population possesses higher; NS formation activity, secondary NS formation, multipotency, and Hes1/Hes5 expression. Interestingly, both high and low CBF1 cells are CD133 positive. Mizutani et al. [2007] argue that the CBF1 activity and consequent upregulation of Hes1 and Hes5 are important features of NSCs.

CLINICAL APPLICATION'S OF NSCS

The use of fetal brain tissue in transplantation therapy scenarios of Parkinson's disease stimulated immense interest in the possibility that NSCs may hold a key to treatment of degenerative diseases of the CNS. The idea of cellular therapy is very appealing and easy to grasp; replace old neurons for new (for a review of cell therapy approaches to CNS repair, see Lindwall et al., 2004). However, the practicalities of such approaches for the treatment of CNS diseases are far from simple. Firstly, we need to generate enough cells of the appropriate cell-type and function. Secondly, we must consider the degenerating environment and how that would affect the survival of introduced cells. Thirdly, and most importantly, whether the newly introduced neurons would send out neurites and synapse at the appropriate places to regenerate circuitry. To date there is little evidence that new neurons derived from NSCs can regenerate circuitry. A more likely role for NSCs in transplantation is to act as support cells providing neurotrophic factors and reversing some of the damage caused by injury and disease [Redmond et al., 2007]. For example, some researchers have bypassed the use of NSCs altogether and used GDNF implants as a means to treat Parkinson's disease [Patel et al., 2005].

So what is the current state of the field of clinical application of NSCs? One criterion for judging the status is to see whether any clinical trials are in progress using these cells. To the authors' knowledge there are only two clinical trials using NSCs ongoing. The first is aimed at treating the devastating degenerative disease of childhood—Batten's disease. The idea here is that the transplanted NSCs will partially supply the missing enzyme thought to be the cause of Batten's disease [Choi, 2007]. The second is aimed at using myc-immortalized human NSC for the treatment of Stroke [Choi, 2007].

FUTURE DIRECTIONS

The study of NSCs is in its infancy. Therefore there are many important questions in NSC biology that need to be tackled. Here I highlight three areas that I think will yield information of immediate relevance to the biology and clinical study of NSCs.

NSC MARKER IDENTIFICATION USING SINGLE CELL ANALYSIS

One of the most critical issues for using NSCs for academic and clinical use is to be able to identify and purify homogenous populations of NSCs. For this goal, ultimately, we need to identify cell surface markers (and antibodies for the marker) that allow us to purify NSCs. A number of markers have been associated with NSCs and these include, CD133/prominin [Uchida et al., 2000], nestin [Rietze et al., 2001], side-populations [Hulspas and Quesenberry, 2000], Lewis-X [Capela and Temple, 2002] and cell size [Kim and Morshead, 2003]. One possible approach to the marker problem is to use "stemness" proteins as potential markers of NSCs and then identify cell surface markers that co-segregate. For embryonic stem cells the transcription factors Sox2, Oct4, and nanog are examples of "stemness" proteins. The recent demonstration that Sox2/Oct4 are the major drivers of induced pluripotency confirms the central importance of these "stemness" proteins in maintaining the stem cell state [Takahashi and Yamanaka, 2006]. Candidate "stemness" proteins for NSCs include; TLX [Shi et al., 2004], Bmi-1 [Molofsky et al., 2003], and Sox2 [Bylund et al., 2003; Graham et al., 2003].

The NS is composed of a heterogenous mix NSCs, NPs, and more differentiated cells. Interrogating the mRNA of this mix of cells complicates the identification of NSC markers. Single cell analysis provides a solution to this problem where distinct populations of cells within NS can be followed and identified. Thus mRNA expression analysis of single cells in combination with co-segregation of "stemness" proteins with cell surface markers will allow us to identify markers for NSCs.

HIGH-THROUGHPUT SCREENS

Using the NS culture system it is possible to set-up assays that report self-renewal, proliferation, and multipotency. These assays can then be used, for example, as a basis to do RNAi screens that select for proteins that play important roles in NSC behavior. In the context of NSCs the study of Diamandis et al. [2007] represents an important example of the strength of high-throughput screening to probe NSC cell biology. Using NS formation as an assay Diamandis et al. [2007] screened 1,267 compounds from the LOPAC library. One hundred and sixty compounds were found to inhibit NS formation and interestingly a variety of NS phenotypes were detected including changes in NS size and shape and cell–cell/cell–surface interactions. After the initial screen, 43 candidates were interrogated with 28 compounds going through to detailed analysis that included dose-response affects on a mouse astrocyte cell line. The main conclusion of the LOPAC library screen was that neuromodulators (agonists/antagonists that affect Dopamine, NMDA, Opiod, Serotonin, and Vallinoid receptors) inhibit NS formation/astrocyte growth and may be candidates for anti-cancer treatment. Since neuromodulators are already in current clinical use they represent good candidates from for further clinical assessment. Finally, Diamandis et al. [2007] speculate that the high intake of neuromodulators by Parkinson's disease patients may explain the low incidence of brain cancer found in this group.

MODELING CNS DEVELOPMENT

Cells from NS culture can be induced to differentiate by changing the environment of the cells. Typically, to induce differentiation of NSCs, cells are plated on laminin, EGF/FGF are withdrawn, and serum added. Under laminin/serum induced differentiation the predominant cell type is astrocytes, followed by neurons and a low frequency of oligodendrocytes. If neurotrophic factors such as NGF, BDNF, and GDNF are added during differentiation the numbers of neurons increases significantly at the expense of astrocytes. If platelet derived growth factor, via Sato's medium, is present the number of oligodendrocytes dramatically increases to make up the dominant cell type (Fig. 2). Thus it is clear that cells derived from NS are plastic in their differentiation potential and by changing the media composition the proportion of the three neural cell types can be altered.

Plating whole NS under differentiating conditions generates a mixed culture of at least three distinct cell types. In essence the differentiating NS can be seen as a piece of CNS tissue. Following time-lapse analysis it emerges that neurons are seen at the leading of

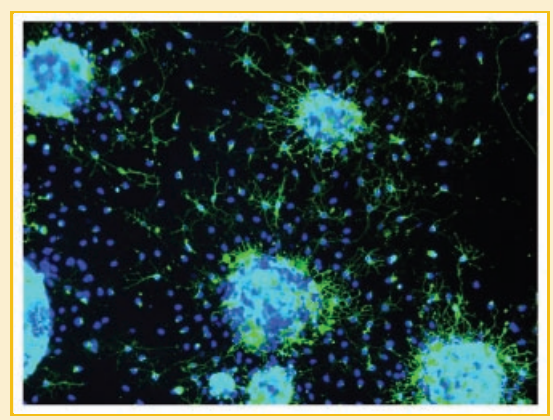

Fig. 2. Differentiated NS showing the formation of oligodendrocytes. NS are plated on laminin, and then fixed and stained for O4 to mark for oligodendrocytes (green) and the nucleus is stained blue.

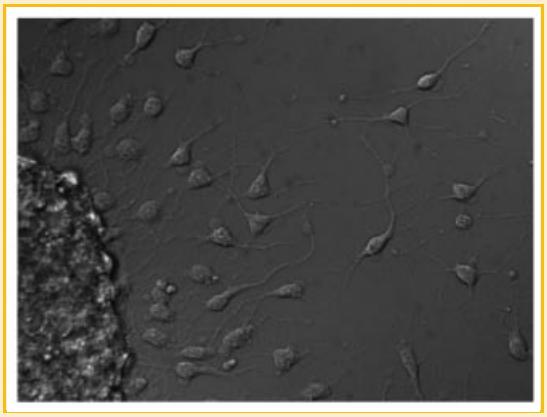

Fig. 3. Morphology of cells emerging from NS. A DIC image of a neurosphere edge is shown. NS are plated on laminin under differentiating conditions. Neurons and oligodendrocytes but not astrocytes can be seen emerging from the leading edge. Astrocytes remain within the NS.

the NS migrating out on to the matrix (Fig. 3). Neurons are followed by oligodendrocytes and lastly astrocytes, which generally remain within the sphere. This sequence of cell type emergence recapitulates events occurring during neurodevelopment. It would be interesting to see if neurons within the culture system form synapses and electrical connections, or if the oligodendrocytes interact with neurons, or whether wound healing experiments induce astrocytes to generate factors such as cytokines and proteoglycans. Critical to using NS and NSCs as models for neurodevelopment will be the establishment of cell lines. Although mouse cells passage reasonably well it would be advantageous to be able to immortalize and propagate NSCs as well as NPs that have made lineage commitments. For example, the immortalization of early, mid, and late oligodendrocyte precursor cells would be important for following development of this lineage. Table I presents some of the NSC lines that have been developed for both mouse and human. The generation of new cell lines including ones that carry mutations linked with CNS diseases will provide a crucial resource for further study. The potential of generating patient specific cell lines through induced pluripotency strategies is also on the horizon.

These three highlighted future directions are not mutually exclusive and it is likely that they will have impact on each other.

TABLE I. Table of NSC Lines

Cell line	Species	Immortalization	References
MHP36	Mouse	ts SV40 T antigen	Gray et al. [1999]
C17.2	Mouse	Myc	Snyder et al. [1992]
CD133 selected	Human	Primary cells[a]	Uchida et al. [2000]
HNCS.100	Human	Myc	Villa et al. [2000]
HB1.F3	Human	Myc	Kim [2004]
ReNCellVM/ReNCellCX	Human	Myc	Sinden [2006]
hTert	Human	Retroviral hTert	Roy et al. [2007]
IhNSC	Human	Myc	De Filippis et al. [2007]
ReN001/ReN005	Human	Inducible Myc[b]	Miljan et al. [2008]

A range of mouse and human NSC lines is shown with the mechanism of immortalization and sources.
[a]These cells are in clinical trials for Batten's disease.
[b]These cells are in clinical trials for Stroke (for review see Choi, 2007).

For instance, cell lines will be useful to standardize conditions for compound, drug, and RNAi screens. In conclusion, there is no doubt that the study of NSCs will provide a wealth of information about the CNS as well as ways to treat CNS diseases. Establishing a strong platform of knowledge on NSC biology will be a crucial stepping stone in realizing the potential of NSCs.

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