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Review article

Isolation, culture and analysis of adult subependymal neural stem cells

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ABSTRACT

Individual cells dissected from the subependymal neurogenic niche of the adult mouse brain proliferate in medium containing basic fibroblast growth factor (bFGF) and/or epidermal growth factor (EGF) as mitogens, to produce multipotent clonal aggregates called neurospheres. These cultures constitute a powerful tool for the study of neural stem cells (NSCs) provided that they allow the analysis of their features and potential capacity in a controlled environment that can be modulated and monitored more accurately than *in vivo*. Clonogenic and population analyses under mitogen addition or withdrawal allow the quantification of the self-renewing and multilineage potency of these cells and the identification of the mechanisms involved in these properties. Here, we describe a set of procedures developed and/or modified by our group including several experimental options that can be used either independently or in combination for the *ex vivo* assessment of cell properties of NSCs obtained from the adult subependymal niche.

Contents

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1. Introduction

Adult mammalian stem cells support tissue renewal and are endowed with fascinating capacities, which allow them to replicate themselves while producing differentiated progeny during the lifespan of the organism. Despite their remarkable potential, these cells are generally present in small numbers and can be relatively quiescent and, therefore, their identification and analysis have heavily relied upon their isolation from the tissue that they inhabit. Some adult stem cells, such as hematopoietic stem cells, can be identified prospectively by surface markers, isolated by fluorescent-activated cell sorting and directly transplanted *in vivo* for the analysis of their fundamental properties (Kondo et al., 2003). In contrast, other stem cells have been isolated by culturing tissue dissociates under conditions that promote their selective expansion (Daynac et al., 2015; Ferron et al., 2007; Mich et al., 2014; Pastrana et al., 2009; Reynolds and Weiss, 1992). Although the latter approach only grants an operational definition of a stem cell, the establishment and optimization of culturing conditions for distinct populations of adult stem cells have notably increased our knowledge on how these cells are regulated, for example by signals from their microenvironment or niches (Porlan et al., 2013b).

In the mammalian brain, ongoing production of new neurons and oligodendrocytes supported by neural stem cells (NSCs) continues after birth in two restricted germinal areas, the subgranular zone (SGZ) of the dentate gyrus in the hippocampus, which produces interneurons that become integrated within the same structure, and the far more active subependymal zone (SEZ; also called ventricular–subventricular zone, or V–SVZ), located adjacent to the striatum in the walls of the lateral ventricles, which generates olfactory bulb interneurons and, to a lesser extent, corpus callosum oligodendrocytes (Bjornsson et al., 2015; Bond et al., 2015). In the intact adult SEZ NSCs, or B cells, are relatively quiescent radial glia/astroglia-related glial fibrillary acidic protein $(GFAP)^+$ cells that give rise to transit amplifying progenitor (TAP) cells which rapidly divide a few times before differentiating into neuroblasts or oligodendroblasts (Bjornsson et al., 2015; Bond et al., 2015). Ontogenetically, they derive from neuroepithelial and radial glia cells that act as NSCs during fetal development. After birth, most radial glia cells differentiate into astrocytes but some of them persist as NSCs in the above mentioned neurogenic niches (Kriegstein and Alvarez-Buylla, 2009).

Initial work by Reynolds and Weiss led to the establishment of defined culture conditions that allowed the isolation and expansion of individual cells from young adult (2-month old) mouse periventricular tissue under non-adhesive conditions. These cells were initially maintained in a serum-free medium containing epidermal growth factor (EGF) to induce their proliferation. Under these culture conditions most of the cells died during the first days in culture, but a small population of cells began to divide and formed floating aggregates of cells with immunocytochemical features of neuroepithelial cells, called primary "neurospheres" (Reynolds and Weiss, 1992). Subsequent mechanical dissociation and subculture of these neurospheres allowed propagation of the cultures, revealing the self-renewal capacity of some of the cells. Additionally, when cultured onto an adhesive substrate in the presence of serum, they could produce both astrocytes and neurons. This provided the first *in vitro* evidence that multipotential stem cells were present in the adult mammalian brain and a method to expand large numbers of postnatal NSCs (Reynolds and Weiss, 1992).

Since these early experiments, the neurosphere culture has evolved into a powerful tool that enables the study of NSC proliferation, self-renewal and developmental potential under highly controlled environmental conditions. However, this type of culture has some limitations. The most important one is that neurosphere cultures contain a heterogeneous population of cells as NSCs coexist with their progeny (different types of more committed progenitors and even differentiated cells). Stem cells unavoidably produce cell progeny *in vitro* and some of the highly proliferative committed progenitors or TAP cells appear also capable of forming neurospheres, albeit only for a few passages (Doetsch et al., 2002; Reynolds and Rietze, 2005). Indeed, only a fraction of the total cells in the culture behave as *bona fide* NSCs and, therefore, the assays need to carefully address their specific properties. In addition, recent evidence indicates that quiescent and actively dividing (activated) stem cells coexist in adult stem cell niches (Codega et al., 2014). Prospective isolation of pure NSCs from the SEZ neurogenic niche using recently developed combinations of surface markers and fluorescent reporters indicate that quiescent NSCs are a key component of the neurogenic niches and coexist with activated, albeit slowly dividing NSCs and that the neurosphere assay promotes the selective expansion of activated NSCs under strong mitogenic stimulation (Codega et al., 2014; Llorens-Bobadilla et al., 2015; Mich et al., 2014; Pastrana et al., 2009). These isolation techniques still face severe limitations such as the requirement of transgenic reporter mice combined with multiple immunostainings, the low numbers of recovered cells, even after pooling tissue from different mice, and the reduced survival of cells after the sorting process. However, the application of NSC culture protocols to these isolated populations appears as a promising strategy to further understand the fundamental properties and behavior of different types of NSCs.

Potential culture-derived deviations from the features (signaling receptors, metabolic parameters, cell lineage-specific molecules, adhesive interactions,…) that these cells exhibit *in vivo* are also an *in vitro*-inherent limitation that has to be kept in mind.