Chapter 1

Systems for *ex-vivo* Isolation and Culturing of Neural Stem Cells

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1. Introduction

During neural development, a relatively small and formerly considered homogeneous population of Neural Stem cells (NSCs) gives rise to the extraordinary complexity proper of the Central Nervous System (CNS). These represent populations of self-renewing multipotent cells able to differentiate into a variety of neuronal and glial cell types in a time- and region-specific manner throughout developmental stages and that account for a weak regenerative potential in the adult brain [1].

In the adult mammalian CNS, the presence of NSCs has been extensively investigated in two regions, the SVZ and the SGZ of the hippocampus, two specialized niches that control NSCs divisions in order to physiologically regulate their proliferative (symmetrical divisions) *vs* differentiative fate (asymmetrical divisions) [2].

In the early '90s it was shown that NSCs could be extracted from the developing and adult mammalian brain and expanded/manipulated/differentiated *in vitro* (Fig. 1).

This has represented a key step in the field, since the obtainment of *in vitro* NSC systems has been very useful in the last years in order to progress toward disclosure of the complex interplay of different extrinsic (signaling pathways) and intrinsic (transcription factors and epigenetics) signals that govern identity and functional properties of brain tissue-specific stem/progenitors [3]. Furthermore, it will also be a key step towards their exploitation for a better dissection of the molecular processes occurring in neurodegeneration [4]. Finally, NSC systems might represent major tools for the potential development of new cell-based and pharmacological treatments of neurodegenerative disorders and for assaying their toxicological effects [5].

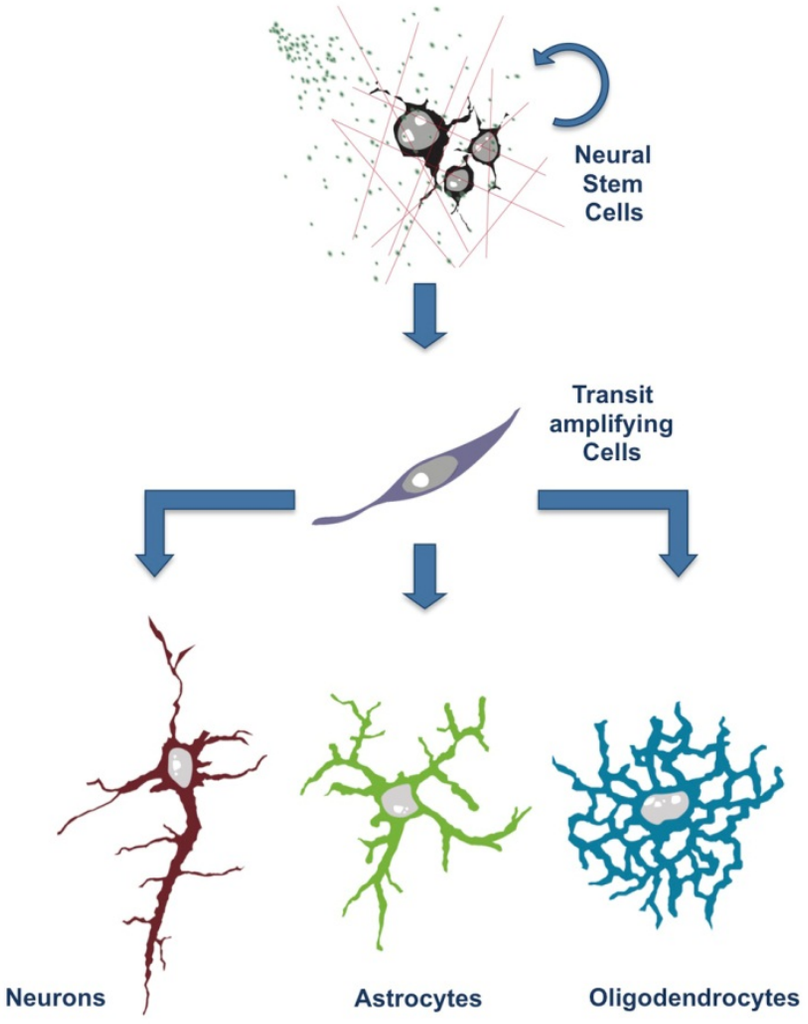

Figure 1. Process of NSC self-renewal and differentiation. NSCs are tri-potent cells. These cells during the differentiation process give rise to transiently dividing progenitors (transit amplifying progenitors) that subsequently undergo lineage restrictions toward neuronal, astrocytic and oligodendroglial mature cells.

Here we will review the functional properties of different *in vitro* NSC systems, providing also a direct comparison with NSCs present *in vivo*. Furthermore, we will discuss some of recent advancements in the development of *in vitro* systems that try to re-create *in vitro* some of the aspects of the physiological NSCs niches.

2. *In vivo* and *in vitro* developmental heterogeneity of NSCs populations

Vertebrate neural development starts with the process of neural induction, during and after gastrulation, which allows the formation of NEUROECTODERM from the dorsal-most part of the ectoderm. The molecular nature of the inductive signals that drive this process has been unveiled by studies in *Xenopus laevis*. These have shown that neural differentiation is promot-

ed by secretion of an array of BMP inhibitors, chordin, noggin and follistatin produced by an embryonic structure called "organizer" [6, 7]. The organizer also produces inhibitors of the Wnt signaling pathway, such as Dickkopf, frzb and cerberus [8]. Neural induction has shown a remarkable evolutionary conservation and a "default" model has been proposed, which states that ectodermal cells have an intrinsic predisposition to differentiate into neuroectoderm, unless inhibited by BMP signaling [9]. While in certain conditions this seems to be the case, in other assays positive inducers are needed, such as FGFs [10]. Finally, more recent studies show that inhibition of Activin/Nodal pathways also seems to be important for neural induction [8].

Progresses in cell culture technologies combined with a better understanding of these developmental progressions have allowed now to recapitulate these processes *in vitro* through neuralization of mouse and human pluripotent cells, i.e. Embryonic Stem cells derived from blastocyst stage (ESC; [11]) and reprogrammed cells (iPSC; [12, 13]), leading to the generation of populations of EARLY NEUROEPITHELIAL CELLS (Fig. 2). These cells give rise to all of the neural cells in the mature CNS thus denoting their extensive multipotential aptitude in terms of different cellular subtypes they can produce. Sox1 is the earliest identified marker of neural precursors in the mouse embryo and is present in dividing neural precursors from the NEURAL PLATE and NEURAL TUBE stages [3]. Studies on pluripotent cells support the "default" model for mammalian neural induction. *In vitro* studies have in fact shown that during neuronal differentiation, ESCs and iPSCs undertake gradual lineage restrictions analogous to those observed through *in vivo* fetal development, and a variety of distinctive progenitors can be generated. Accordingly, mouse and human pluripotent cells differentiate into sox 1 positive neuroepithelial cells (note that in human the earliest neuroepithelial marker is represented by pax6 that precedes sox1 expression) when grown in serum-free conditions in the absence of patterning signals [14-16]. ESCs and iPSCs neural induction can be enhanced by the addition of BMP-, Nodal- and Wnt-inhibitors, to minimize endogenous signals produced by ESCs/iPSCs themselves and recent studies have shown that paracrine signals (i.e. FGF4) are also needed for neurulation [17, 18].

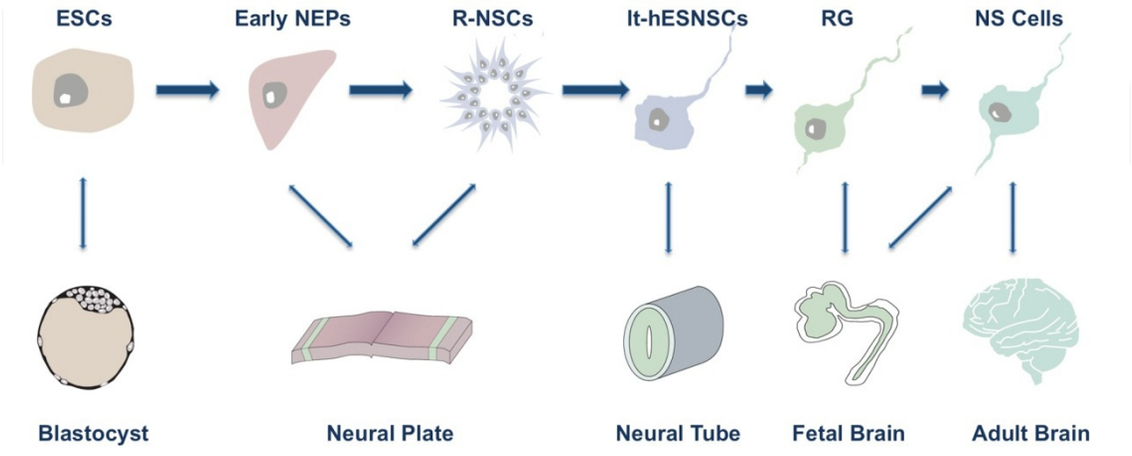

Figure 2. The different NSC populations that can be obtained in vitro correspond to stage-specific neural progenitors present at defined in vivo developmental stages.

Soon after neural induction process, pluripotent cell-derived neuroepithelial cells give rise to NEURAL ROSETTE structures, in which cells elongate and align radially, in a manner that mimics neural tube formation [19]. *In vivo*, the neural tube is formed after neurulation from the newly-induced neural plate and, as it closes, it is regionalized along the antero-posterior (A/P) axis (Fig. 3A), giving rise to four main areas: forebrain, midbrain, hindbrain and spinal cord. In amniotes, dorso-ventral (D/V) patterning takes place only after A/P patterning has occurred, after neural tube closure. The variety of neuronal cells that will be generated will have specific functions according to their position along these two axes.

Several evidences suggest that primary neural induction obtained by BMP inhibition generates anterior neural tissue, while to obtain tissue with posterior characteristics other molecules, known as "transformers", are needed. Three molecules with posteriorizing activities are known: retinoic acid (RA), Fgfs and Wnts [20, 21]. These signals are produced by the surrounding axial and paraxial mesoderm and endoderm, in addition two secondary signaling centers exist within the neural tube [22]. These are the Anterior Neural Ridge (ANR), located at the border between the forebrain and the non-neural ectoderm, and the isthmic organizer, located at the mid-hindbrain boundary. The ANR secretes the organizer molecules noggin and chordin, the resulting BMP signaling inhibition activates Fgf8, which in turn induces the expression of the transcription factor FoxG1 (Bf1), necessary for forebrain development [23]. The isthmic organizer is located at the boundary between the expression domains of the transcription factors Otx2 and Gbx2, and it is formed and maintained by an intricate regulatory network among these and other (En1/2, Pax2/5/8) transcription factors. The isthmic organizer secretes Fgf8, and the feedback loop that is set up assures the maintenance of the tissue identity [22]. RA and Wnts are produced by paraxial mesoderm with a high-posterior/low-anterior gradient and they are responsible for the patterning of midbrain, hindbrain and anterior spinal cord. Among the genes differentially regulated by varying concentrations of RA are the Hox genes, necessary for hindbrain and spinal cord A/P patterning [24, 25]. D/V patterning is mediated by signaling molecules secreted by the surrounding tissues (Fig. 3B). The overlying ectoderm produces TGFβ-family molecules that promote the formation of the roof plate in the dorsal neural tube, while the underlying notochord secretes SHH, that induces the ventral neural tube to become the floor plate. The roof plate and the floor plate in turn become a source of TGFβ and SHH, respectively. This creates two opposing gradients that provide positional information along the D/V axis, regulating the expression of key transcription factors. These will then act in a combinatorial manner to regulate the differentiation of specific neuronal and glial cell types in the correct position [26].

These *in vivo* studies have ultimately revealed that different neural progenitor populations can exist in a time and space-dependent manner and that their fate is greatly influenced by the interplay between specific extrinsic and intrinsic signaling molecules. ESCs- and iPSCs-derived neuroepithelial cells are able to perceive the positional information of patterning signals. These progenitors, when obtained in conditions that minimize endogenous signals, intrinsically acquire anterior identity, while they can be caudalized by the addition of FGFs, Wnts, RA [1, 19, 27, 28].

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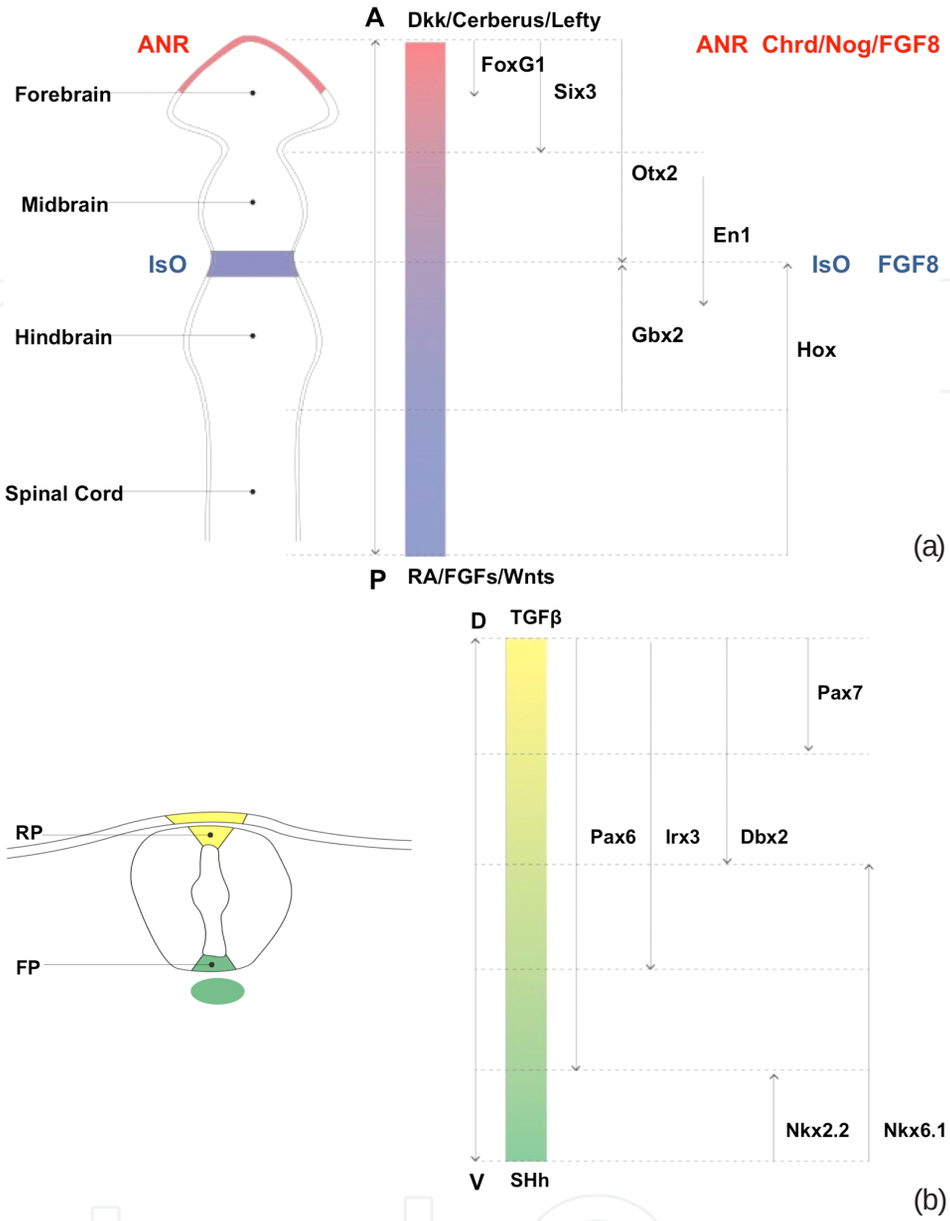

Figure 3. Regional patterning of the neural tube. Schematic diagrams showing antero-posterior (A) and dorso-ventral (B) patterning of the neural tube. The patterning process is driven by opposing gradients of signaling molecules that induce the expression of region-specific transcription factors in discrete areas. ANR: anterior neural ridge. IsO: Isthmic organizer. RP: roof plate. FP: floor plate.

Some studies have shown that NEUROEPITHELIAL CELLS cannot be maintained *in vitro* by the exposure to commonly used mitogens, i.e. basic fibroblast growth factor (FGF-2) and epidermal growth factor (EGF). These indeed convert these cells into radial glia populations characterized by a limited potentiality in neuronal sub-types they can give rise to. Nonetheless, it has been shown that a neuroepithelial population that grows in rosette-like structures (termed "R-NSCs") can be generated *in vitro* from human and mouse pluripotent cells when exposed to SHH/FGF8 signalling coupled to a N-Cadherin/Forse-1 cell sorting-based protocols [19]. These cells can be maintained *in vitro* for some passages by exposure to SHH and Notch

agonists while showing a rostral $BF1^+$ neuroepithelial identity evocative of the signalling that *in vivo* are required for the induction of the anterior neuroepithelium. R-NSCs are characterized by a comprehensive differentiation potential toward CNS and PNS fates, supporting the idea that the R-NSCs represent neural precursors of the neural plate stage.

Another population of hESC-derived Sox1 positive self-renewing neuroepithelial cells named "lt-hESNSCs", has been described [29]. These cells can be grown as a nearly homogeneous population exhibiting clonogenicity and stable neurogenic potential. Remarkably, they can be maintained for many *in vitro* passages in the presence of FGF-2 and EGF and they preserve some properties of the R-NSCs, such as rosette-like growth, the expression of Bf1 and sensitivity to instructive signals that stimulate their conversion into distinct neuronal subpopulations. Molecular analyses have shown that lt-hESNSCs partly maintain rosette properties, possibly embodying an intermediate developmental stage between rosette-organized neuroepithelial cells and radial glia (see below).

As development proceeds, neuroepithelial cells lose sox1 expression and convert themselves into another transitory stem cell type, the so-called "RADIAL GLIA" (RG). This rapidly constitutes the main progenitor cell population in late development and early postnatal life while disappearing at later postnatal and adult stages [30, 31]. Large numbers of RG cells are found in primary cell cultures from dissociated E10.5-18.5 CNS tissue. Different populations of RG, characterized by lineage heterogeneity, with both regional and temporal varieties, give rise to sequential waves of neurogenesis, gliogenesis and oligodendrogenesis that build up the CNS. The *in vivo* developmental heterogeneity of RG has been also revealed by *in vitro* primary cultures studies that have shown a temporal constraint from neurogenesis to gliogenesis from RG isolated at initial or later developmental periods, respectively [32, 33].

The transition of neuroepithelial cells to RG cells is well recapitulated *in vitro* during neural differentiation of pluripotent cells. RG populations can be efficiently generated from ESCs/iPSCs using differentiation protocols that differ in major aspects between them. Bibel and collaborators generated transient (not expandable) populations of homogeneous RG cells that mature into glutamatergic neurons, as occurring during cortical development [34]. A different population of ESCs/iPSCs-derived RG cells can be obtained by exposing neuroepithelial cells to EGF and FGF-2. These rapidly lose Sox1 expression and acquire RG markers as BLBP and RC2 giving rise to RG-like cells which can be long term expanded in monolayer and at homogeneousness [35]. This conversion is dependent on Notch activity and on the exposure to EGF and FGF-2 [19, 35]. These self-renewing RG cells (called "NS cells") retain the marker signature of RG and the full capacity for tri-lineage neural differentiation, although their neuronal differentiation is limited to the GABAergic lineage [36-38]. These results indicate that pluripotent cells can be differentiated into distinct subtypes of RG – a non self-renewing type with aptitude to generate glutamatergic neurons, and a subtype that self-renews and exhibits a GABAergic differentiation. Such radial glial subtypes can also be found in the developing CNS *in vivo* although RG expansion *in vivo* is restricted to a defined time window.

Along with RG, a further immature population of cells with neuronal-restricted potential is represented by the BASAL PROGENITORs (BPs) that are located in the subventricular zone (SVZ) and can be generated both by neuroepithelial cells and RG [39, 40]. *In vitro* studies on

BPs are less comprehensive. Transitory induction of neurogenic Tbr2-positive BPs has been reported during the differentiation of ESCs to glutamatergic cortical neurons [27]. It has also been shown that BPs can be isolated from a subgroup of RG populations characterized by a high immunoreactivity for prominin that can make neurons only indirectly through the generation of BPs [41].

At the end of neurogenesis (in mice approximately at birth), neurogenic RG cells are exhausted and the remaining RG convert into astrocytes. The presence of stem cells has been reported in two regions of the adult mammalian brain, the SVZ and the SGZ of the hippocampus. Fate-mapping studies have shown that these adult NSC populations are represented by the type B astrocytes that directly derive from subpopulations of fetal RG cells. Therefore, RG and type B astrocytes appear to form a continuous lineage with stem cell potential [2]. These *in vivo* studies find a parallel indirect proof from the fact that *in vitro* adult-derived NSCs reacquire fetal characteristics, such as radial glia markers.

3. In vitro systems for NSCs isolation and expansion

The study of different types of stem cells has greatly beneficed from *in vitro* approaches that allow the reduction the intrinsic complexity of tissues. In order to allow stable maintenance *in vitro*, cells have to be immortalized, a procedure that blocks the progression of developmental programmes by pushing the cells to remain in enduring proliferation. Immortalization can be achieved by means of various methods, most usually by viral transduction of immortalizing oncogenes such as c-myc or SV40 Large T Antigen. Several immortalized murine and human NSC lines have been reported and, interestingly, it has been shown that they maintain many equivalences to non-immortalized lines, exhibiting neglectable signs of transformation both *in vivo* or *in vitro* [42-45]. Nevertheless, the physiological relevance of these lines might be weakened by the expression of potentially transforming oncogenes.

In the developing CNS, exponential cell division occurs only for brief developmental windows and NSCs represent transient populations. In the brain, NSC division is rigorously regulated by many factors of the "NICHE". The niche represents the particular cellular microenvironment that provides the appropriate milieu to support self-renewal and that controls the balance between symmetrical proliferative (producing two stem cells) and asymmetric cell divisions (generating one stem cell and one committed progenitor). Accordingly, for a stem cell to give rise to a clonal cell line, the physiological hindrances to continuous cell division have to be bypassed. However, until few years ago, it has been extremely difficult to stably propagate homogenous cultures of NSCs without oncogene-mediated immortalization procedures.

In the last two decades, oncogene-free procedures based on the use of soluble factors for selection and expansion of NSCs have been developed, permitting long-term maintenance of NSCs. The first report was from Reynolds and Weiss that in 1992 showed that the fetal and adult rodent brains contain cells competent for continuing *ex vivo* prolifera-

tion upon exposure to EGF and FGF-2 and that upon mitogen withdrawal exhibit tri-neural lineage differentiation [46, 47]. According to this procedure, freshly dissociated SVZ cells plated at low density (roughly 10^3-10^4 cells/cm^2) in the absence of cell adhesion substrates and in presence of EGF and/or FGF-2 have the tendency to loosely adhere to the plastic plate. Within few days, most of the cells die except a minor fraction of them that become smooth-edged and begin to proliferate while staying attached to the plate. Later, the progeny of these proliferating cells stick to each other forming sphere-shaped clones that detach from the plate thus floating in suspension giving rise to the so-called NEUROSPHERES. This assay, named "Neurosphere Assay" has thus been widely considered as a valuable method for isolating, enriching and maintaining embryonic and adult NSC populations *in vitro* [48]. Indeed, whereas NSCs in culture are characterized by the ability to considerably divide and self-renew thus giving rise to long-term expanding NSC lines, transit amplifying progenitors exhibit partial proliferative competence without self-renewal potential, and are eliminated during extensive sub-culturing. Notably, only a fraction of cells composing the neurosphere (commonly 1-10% for optimal cultures, although this value greatly differs depending on the age and on the brain area considered) are true stem cells, the remainder being differentiating progenitors at different stages, and even terminally differentiated neurons and glia [49]. Neurospheres can be sub-cultured by mechanical or enzymatic dissociation and by re-plating under the identical *in vitro* settings. As for the primary neurosphere culture, at every sub-culturing passage, differentiating/differentiated cells are supposed to die while the NSCs divide, generating secondary spheres that can then be further sub-cultured [50]. This procedure can be serially reiterated and, since each NSC gives rise to many NSCs by the time a neurosphere is generated, it ends in the expansion of the NSC population in culture.

Once established, neurosphere cultures can be expanded to obtain large amounts of cells that can then be cryopreserved. This permits the creation a pool of cells that can be later thawed and expanded for future experimentations. Nonetheless, several studies have shown that after few passages, the neurospheres greatly decrease their efficiency in neurogenic differentiation [51] and in the neuronal subtypes they can give rise to, mostly restricting their potential to the GABAergic lineage [52] (Fig. 4).

The accurate identification of the identity of the sphere-forming cell represents a key question. As committed progenitors are capable of only restricted proliferative capability and can generate only up to tertiary neurospheres, actually the designation of a cell as *bona fide* NSC should be retrospectively refereed only to a founder cell that self-renews extensively and can be propagated in long-term cultures. To this regard, it has been suggested that at least five sub-culturing passages are required to exclude the contribution of committed progenitors to the maintenance of the cell population. More rigorously, the assay should be performed with single dissociated cells (i.e. to plate a single cell per well) in order to avoid cell clustering and also fusion between neurospheres [53, 54].

Some researchers consider that three-dimensional organisation and the cellular milieu of the neurosphere as the *in vitro* equivalent of the *in vivo* neurogenic compartment [55, 56]. Although this view is a pure speculation, it is broadly accepted that the issue of the complexity of the

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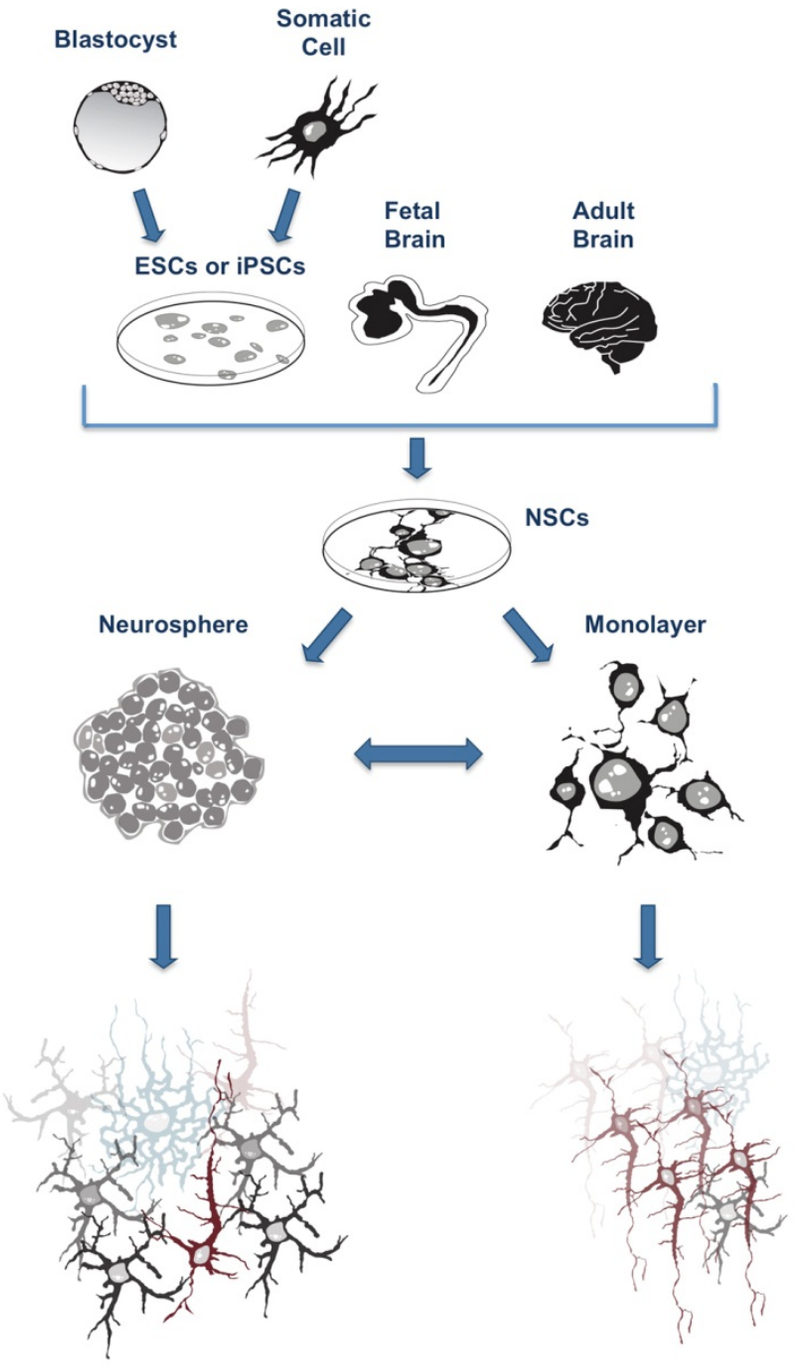

Figure 4. Neurospheres and monolayer NSCs can be obtained by different sources and have different neuronal differentiation efficiency. NSCs grown in monolayer and neurospheres can be derived from ESCs or iPS cells and from the germinative areas of the fetal and adult brain. The homogenous cellular composition of the NSCs grown in monolayer results in a higher neurogenic potential than neurospheres

neurosphere system represents a barrier for fine biochemical and molecular studies. The prospect of refining the neurosphere culture and of developing alternative *in vitro* systems, not only to enrich but also to select and clonally expand the *bona fide* stem cell population

without losing the original prevalent neuronal fate, has been a recurrent issue in the stem cell field.

As an alternative to the neurosphere system, other researchers have developed monolayer-based methods [57]. In 1997, Gage and colleagues reported that progenitor cells with properties similar to NSCs from adult SVZ could be obtained from the adult hippocampus [58]. These hippocampal precursor cells propagate in monolayer and using *in vitro* procedures similar to the ones used for SVZ NSCs. Hippocampal precursors divide in response to FGF-2 and show tri-neural potential being able to differentiate into astroglia, oligodendroglia, and neurons *in vitro*. More recently, the optimization of novel and efficient strategies for the derivation and stable long-term propagation of NSCs from developing and adult neural tissue and from pluripotent cellular sources has been reported. It has been shown that transiently generated ESC-derived neural precursors, normally destined to differentiate to neuronal and glial cells, can be efficiently expanded as adherent clonal NSC lines in EGF and FGF-2 supplemented medium [19, 35]. In these growth conditions, cells undergo symmetrical division with neglectable accompanying differentiation, while shifting of the cultures to differentiative conditions prompts the cells to efficiently generate mature neurons, astrocytes and oligodendrocytes, thus indicating their NSC essence. The cells obtained by this procedure have been named Neural Stem (NS) cells. Notably, these results suggest that expansion of NS cells can occur in the absence of a complex cellular niche. Accordingly, NS cell expansion in monolayer conditions restrains spontaneous differentiation and permits proliferation of homogeneous *bona fide* NSCs.

Phenotypic characterization of NS cell cultures indicates a close similarity to forebrain RG [35]. Indeed, NS cells are homogenously immunopositive for nestin, SSEA1/Lex1, Pax6, prominin, RC2, vimentin, 3CB2, Glast, and BLBP, a set of markers diagnostic for neurogenic RG. NS cells keep their neurogenic potential after extensive expansion (over 100 passages), yet retaining the capability to produce a large proportion of mature neurons (Fig. 4). These results further indicate that the acquisition of RG properties endows the cells with a "niche" that traps them in a state of symmetric cell division. Significantly, NS cells do not represent a peculiarity of ESCs and iPSCs cell differentiation [35, 59]. In fact, similar lines can also be obtained from foetal or adult CNS and established from long-term expanded neurosphere cultures [35, 60, 61]. It is therefore possible that NS cells embody the resident NSC population within neurospheres. Further characterization of different mouse NS cell lines has demonstrated a close similarity in self-renewal, neuronal differentiation potential and molecular markers, independently from their origin. NS cells are not exclusive for mouse sources but it has indeed described the possibility to generate NS cells both from human fetal neural tissue and from human ESCs [62]. Interestingly, similar cells can be developed also from brain tumors and might serve as systems for find new targets in order to develop new therapeutic approaches [63, 64]. Similarly to NS cells, also lt-hESNSCs grow in monolayer and can be long-term expanded but differently from NS cells, they maintain sox 1 expression and a wide developmental competence [29, 65]. These aspects might be suggestive for some species-specific differences.

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4. Influences of the *in vitro* systems on the molecular and biological properties of NSC lines

For brain tissue, founder NSCs existing during embryogenesis do not endure in adulthood but switch to a quiescent state following completion of development. Therefore, it might be expected that in order to achieve persistent propagation of NSCs *in vitro* it might not be merely sufficient to follow intrinsic programmed mechanisms but also modifications of the "Neural Stem Cells cellular "character" are required to adapt to the synthetic *in vitro* milieu might also be required. Indeed, the interaction of typical transient progenitor populations with the artificial *in vitro* environment (i.e. high levels of growth factor stimulation and/or different matrix or cell-cell interactions) may modify their transcriptional and epigenetic status, allowing them to be "turned" into NSC lines.

In this view, when coming to the nature of the NSCs, the crucial issue is if they do exactly represent a definite sub-population of NSC/progenitor existing *in vivo*. Currently, it is still not entirely understood if the accomplishment of the NSC status might be the effect of phenotypic alterations due to culture set and how physiologically relevant the consequent *in vitro* phenotype might be [3]. Thus, it is preferable to refer to *in vitro* expanded NSCs as NSC-like cells.

To this regard, the possibility that the mixture of mitogens may produce an artificial cell condition with a proper balance of key transcription factors able to suppress lineage commitment and allow self-maintaining divisions has to be considered. It has been shown that FGF-2 and EGF, two growth factors typically used for the *in vitro* maintenance of NSCs can alter the transcriptional and epigenetic phenotype. For example, expression of several genes can be directly stimulated *in vitro* in neural progenitors by exposure to FGF-2, suggesting that these genes might exert fundamental functions in the establishment of NSCs lines [66]. Similarly, foetal neural progenitors *in vitro* exposed to FGF-2, rapidly activate expression of Egfr (ErbB1) and Olig2, the latter being a bHLH transcription factor linked with the oligodendrocyte lineage and ventral CNS identity [66, 67]. Under expansion conditions with high levels of EGF and FGF-2, induction of Olig2 is required for the proliferation and self-renewal of neurosphere cells and NS cells, as demonstrated by analyses in which experimental interference with Olig2 expression severely decreases the amount and the quality of neurospheres [68]. Besides Olig2, it has been shown that acute exposure to FGF-2 induces neural progenitors to upregulate expression of a broad set of genes (for example CD44, GLAST, Olig1, Cdh20, Adam12 and Vav3) likely playing significant roles in the phenotype of the cells [69]. Likewise, EGF has been shown to deregulate expression of Dlx-2 in NS cells, NSC cultures and in transit-amplifying cells of the SVZ, inducing their switch into RG-like neurosphere-forming cells [51, 61, 69, 70]. Remarkably, stimulation of several of these genes (for instance Vav3 and CD44) occurs within few hours of FGF-2 exposure, possibly indicating that mitogen-mediated action is not suggestive for a physiological developmental progress but rather an acute transcriptional rearrangement [69].

NSCs *in vivo* have been shown to be tremendously heterogeneous in terms of transcriptional factors expression pattern, a feature predictable to confer a complex elaboration of positional signals [33]. To this regard, several reports have shown the occurrence *in vitro* of profound variations in the expression pattern of positional genes compared with primary precursors

and progenitors *in vivo* thus leading to a mixed regional identity and limited neuronal differentiation. For example, neurospheres from the spinal cord have been shown to undergo upregulation of Olig2 and downregulation of the dorsal spinal cord transcription factors Pax3 and Pax7 [71]. Olig2 and Mash1 are also induced in E14 cortex or ganglionic eminence precursors, short- or long-term grown as neurospheres [72]. With some exceptions, a similar deregulation of the regional patterning is evident in the adherent NS cells and lt-hESNSCs cultures [29].

Importantly, this relaxation in the positional code might be related to a recurrent restriction in the competence to generate diverse neuronal subtypes. Indeed, NSCs have been reported to rapidly lose their original competence to generate site-specific neuronal subtypes when cultured *in vitro*, both in monolayer and in aggregation, in the presence of EGF and/or FGF-2, becoming mainly constrained to adopt a GABAergic fate [35, 52, 73, 74]. A notable exception is represented by the lt-hESNSCs [29], possibly indicating that for some reasons neuroepithelial cells derived from human pluripotent sources are more "predisposed" to long-term better preserve a broad neuronal sub-types developmental competence.

On the whole, these results might thus emphasize an artificial nature of cell culture, underlining the requirement for prudence in extrapolation of *in vitro* results to normal development or physiology without corresponding *in vivo* data [3]. Alternatively, this might be due to inadequate culture conditions that are not actually competent to preserve the molecular and biological properties of genuine NSCs.

5. Reconstruction of NSC niche *in vitro*

NSC niches present distinctive features leading to diverse ways to ensure neurogenesis. In the adult SVZ, three main immature neural populations lie adjacent to a layer of ependymal cells lining the lateral ventricle wall [2]. The Type B cells, representing the NSCs, reside interposed into the ependymal layer, displaying connections with both the ventricular wall and the blood vessels-network characterizing this niche. They are relatively quiescent but capable of giving rise to transit amplifying cells (Type C cells), a more rapidly dividing population that in turn generate the third population composed by neuroblasts (Type A cells) that migrate into glial tubes to reach the olfactory bulb. Besides these populations, a vital role for the maintenance of the niche is played by ependymal cells (Type E cells), astrocytes and endothelial cells. A comparable organization has been reported also for hippocampal SGZ niche although this exhibits a more planar structure [75, 76]. For a more detailed description of the neurogenic niches refer to of this book.

It emerges that both of these neurogenic niches are arranged to allow NSCs integration and to permit a strict responsiveness to signals from the *"external world"* (blood vessels and ventricles) and the "*neighboring world*" (newly generated neuroblasts, resident astrocytes and microglia, ECM components-forming scaffolds, etc.). All of these components harmoniously interact with each other providing both positive and negative signals and feedback that regulate NSCs activity.

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Even though it is still a long way to fully understand the complex physiological context of a niche, researchers are now trying to reproduce *in vitro* at least some aspects of the dynamic *in vivo* environment. A better comprehension of the mechanisms underlying the NSC niche and the development of systems aimed at the reconstruction of this milieu will fill the gap between bi-dimensional (2D) simplified *in vitro* studies and the complex but physiological conditions of *in vivo* methods.

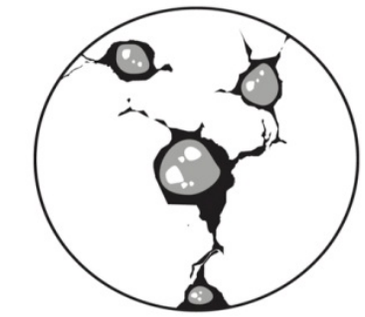
To this purpose, a synthetic NSC niche should recreate the complex interactions between NSCs and others cells, extracellular matrix, gradients of regulatory molecules and physical factors (Figure 5). In particular an ideal *in vitro* mimicked SVZ niche should contemplate the following minimal requirements:

1. presence of NSCs
2. production of the characteristic NSC niche-signaling molecules
3. presence of a basal lamina and extracellular matrix
4. autonomous production of cellular and molecular factors necessary for self-renewal and differentiation of resident stem cells
5. incorporation of extra-neural (i.e. endothelial cells) cells
6. spatial assembly reproducing the SVZ *in vivo* architecture.

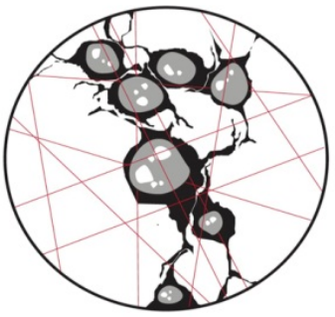
In vitro generation of structures grossly simulating the SVZ NSC niche have been reported from mouse ESC-derived NSCs without the administration of mitogenic factors and complex physical scaffolds. In these studies, following a neuralization process with retinoic acid and plating the NSCs at high density on an entactin-collagen-laminin coated surface, heterogeneous multicellular aggregates appeared spontaneously, showing some of the characteristics postulated above, although a well-defined structural architecture was lacking [77]. In the last years, the development of new 3D culture systems that can allow to better reproduce *in vitro* structures in between standard monolayer culture and living organisms have been/are under investigation.

In this direction, standard culture methods involving petri dishes are being replaced with more accurate micro-scale devices, allowing procedures at the time and length scales of biological phenomena, enabling the control of multiple parameters, such as molecular and physical factors [78]. More attention is now focused on both the generation of morphogen-gradients, taking advantage of microfluidic systems, and three-dimensional extracellular matrix mimic-scaffolds in which multiple cells can be entangled allowing spatiotemporal control of the system and satisfying all of the features of a niche [79].

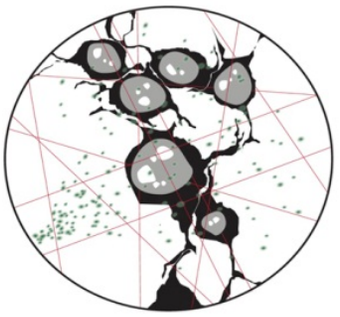
Microfluidic systems can reproduce a niche-like microenvironment permitting also the generation of concentration gradients of signaling molecules, often without the application of an external power source. Indeed, two different solutions can be introduced into the main channel of a microfluidic-chip by an osmotic pump. Since at this scale fluids mix only by diffusion, at the interface of the two solutions, diffusion generates a stable concentration



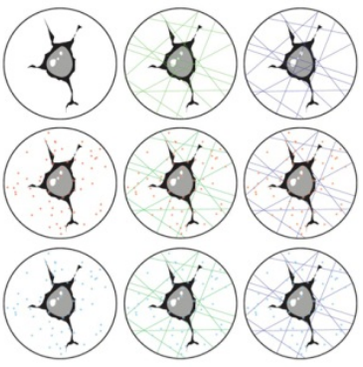
Standard Culture Dishes
- Standardized methods
- Flattened morphology
- High amount of signaling molecules required
- Expensive
- Far from physiology



Synthetic Extracellular Matrix
- More physiological environment
- Possibility to functionalize the matrix
- Expensive
- Cell behavior yet to be best investigated



Microfluidic combined with Synthetic ECM
- Same scale as live organisms
- Lower amount of molecules needed
- Behavior yet to be best characterized
- Difficut to translate pico doses of the chip to micro doses usually implied



High-Throughput Methods
- High number of samples assessable at the same time
- Automatizable
- Careful results validation needed

Figure 5. Schematic illustration of the different colture methods to reproduce *in vitro* the NSC niche.

gradient. To this regard, it has been shown that solutions of SHh, FGF8 or BMP4 are able to induce human ESC-derived NSCs neuronal differentiation, leading to the formation of a complex cellular network [80].

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A fundamental impulse has come from the advance in the field of BIOMATERIALS. These have been greatly improved in the last few years, allowing now to finely control cell-matrix interactions, to direct cell migration and to permit the precise topographical administration of defined physical (both soluble or not) signals.

While it is quite difficult to modify only one variable with a naïve ECM component, the use of biomaterials has improved and simplified many experimental approaches. For example, when using natural matrices, decreasing the concentration of collagen leads to a decrease stiffness of the gel, nonetheless this also determines a decreasing in the concentration of adhesive ligands and an increase in diffusion, resulting in accumulation of variables to the system. This can be avoided with engineered biomaterials that enable isolation of individual variables, without varying others. Nowadays, synthetic biomaterials are greatly exploited to mimic the physical and mechanical features of the ECM. They allow to control a number of important parameters, including polymerization, degradation, and biocompatibility and to combine them with fully defined chemical components [81-87].

Another point of control allowed by new biomaterials is the possibility to incorporate cells releasing molecules or molecules *per se* as soluble factors, such as cytokines, NFs and GFs. Indeed, these molecules are constantly synthesized, secreted, transported, and depleted in NSC niches. To this regard, Zhang and colleagues have described a 16-residues peptide capable of self-assembly into membrane upon addition of a physiological concentration of salt [88]. Now commercially available as PuraMatrix™, it has been shown to support neurite outgrowth and synapse formation [89] and more recently to regulate murine and human NSCs growth and differentiation following adjunction of NSCs-active molecules [90-93].

Synthetic peptides can also be used in combination with a variety of polymers to provide materials with cell-adhesive, enzymatically degradable, and GFs-binding properties. Amino-acid sequences commonly include collagen-, laminin-, and fibronectin-cell-adhesive domains, these can be mixed together and with other bioactive motifs, such as proteolytically degradable sequences, to create a multifunctional peptide material with different physical properties. For instance, NSCs survival has been shown to be improved in a collagen hydrogel that incorporates laminin-derived adhesion motifs [94]. Peptides can also be used as structural components.

The reconstructions of a NSC niche can be translated to multiwell-based high-throughput methods for screening compounds that can positively regulate neurogenesis and thus be developed as potential therapeutic drugs. Protein-based microarrays have been developed and applied to diverse stem-cell populations [95-97]. These devices consist of robotically spotted GFs or ECM molecules in combinations, on cell repellent substrates in order to avoid cell migration, and cell fate changes are often analyzed via immunocytochemistry assays. Platforms like these have been used to analyze human NSCs differentiation and proliferation in response to combinations of ECM components, morphogens and other signaling proteins. A joint effect of Wnt and Notch pathways to maintain human NSCs in an undifferentiated state, a dose dependent activity of Notch ligands in shifting neuronal differentiation towards glial fate and a neurogenic effect of Wnt3A have thus been reported. Consequently, it is

possible to highlight specific responses of single versus combination of stimuli in a high-throughput way [97].

These platforms are limited to adherent cells only and do not allow cell fates determination on single cells. The hydrogel microwell array, developed on micrometer-sized cavities, permits to analyze both adherent and nonadherent cells, trapped by gravitational sedimentation. The device has been used to analyze single cell-forming neurospheres, avoiding the usual merging events of neurosphere assay [98] and more recently it has been combined with robotic protein spotting to address the role of biochemical and biophysical factors on single nonadherent neural stem cell self-renewal [99].

6. Conclusions

Our knowledge of the neural progenitor identity and properties during development has been radically revolutionized by the possibility to isolate and expand NSCs *in vitro*. We have reviewed here the current and most commonly used *in vitro* methodologies to isolate, expand and functionally characterize NSC populations. The real identity and the potential lineage relationships between different types of stem/precursor cells isolated and cultured *in vitro* by these different methodologies represents a field of open and intense investigation.

In light of the complexity of the biological concerns governing stem cell maintenance and differentiation, significant progress will require a close coordination between *in vivo* and *in vitro* approaches. In this scenario, *in vitro* systems of NSCs shall allow a deep analysis at cellular level providing useful information to be further validate in the embryo and adult in order to identify relevance to normal physiology.

Establishment of *in vitro* settings necessarily results in disruption of the three-dimensional tissue structure, loss of specific cell-to cell contacts and modification of the extracellular environment and signaling. This might also lead to alteration of biological and molecular properties and acquisition of stem cell features by committed progenitors. Thus, although the versatility shown by NSC cultures *in vitro* can be envisaged as an advantage, extreme caution is necessary when considering the potential *in vivo* translation to developmental biology.

NSC biology holds tremendous potential for neurological therapy. It should be emphasized that the study of the intrinsic properties of NSCs and understanding the mechanisms of interaction between resident CNS cells and grafted NSCs will be mandatory for the development of new therapies able to slow the progression of neurodegenerative diseases.

Beside the therapeutical applications, NSCs systems present unique opportunities that are starting to be successfully explored for genetic or chemical screens in order to identify and optimize molecules/drugs that may allow a tight control on self-renewal and lineage specification of NSCs as well as their functional maturation, thus moving forward NSCs-based therapies.

We can anticipate that a rigorous characterization of the functional features of the NSC populations isolated and propagated by means of different cell culture systems shall allow us

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to exploit the advantages offered by one method or the other, depending on the goal of our research.

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